Regenerative Nephrology

Second Edition

Edited by
Michael S. Goligorsky

REGENERATIVE NEPHROLOGY

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SECOND EDITION

Edited by

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To my mentors Anatoly Zubkov, Cidio Chaimovitz, and Keith Hruska
and to my former students who are now mentors themselves.

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Introduction

Ten years after the publication of *Regenerative Nephrology*, the second edition of the book has been conceived and completed. This revised and expanded edition has been motivated by the changing landscape of the field of regeneration, which reflects a multitude of discoveries made during the past decade, rotating investigative windmills of regenerative medicine and nephrology. At times, those may seem to be quixotic, but auspiciously, their blades appear to begin harvesting energy from the winds of change.

In accord with the Heraclitus's motto "There is nothing permanent except change," all the chapters in the second edition are written anew or critically revised. Entirely new sections have been introduced in the second edition, such as Paracrine Regenerative Potential of Stem Cells, Cell Senescence and Rejuvenation, Cell Reprogramming, and Cell Reconditioning. The guiding principle of the book, however, has not changed. It is (1) to discern innate elements of repair processes inherent in every pathological event, (2) to enhance them while suppressing pathogenic driving mechanisms, and (3) to capture the established and emerging strategies to regenerate the kidney by endogenous, similar to Aristotelian entelechy, and/or pharmacologic means. In fact, the entire structure of the book could be encapsulated in a single schematic (Fig. 1) that reflects all the aspects of this edition.

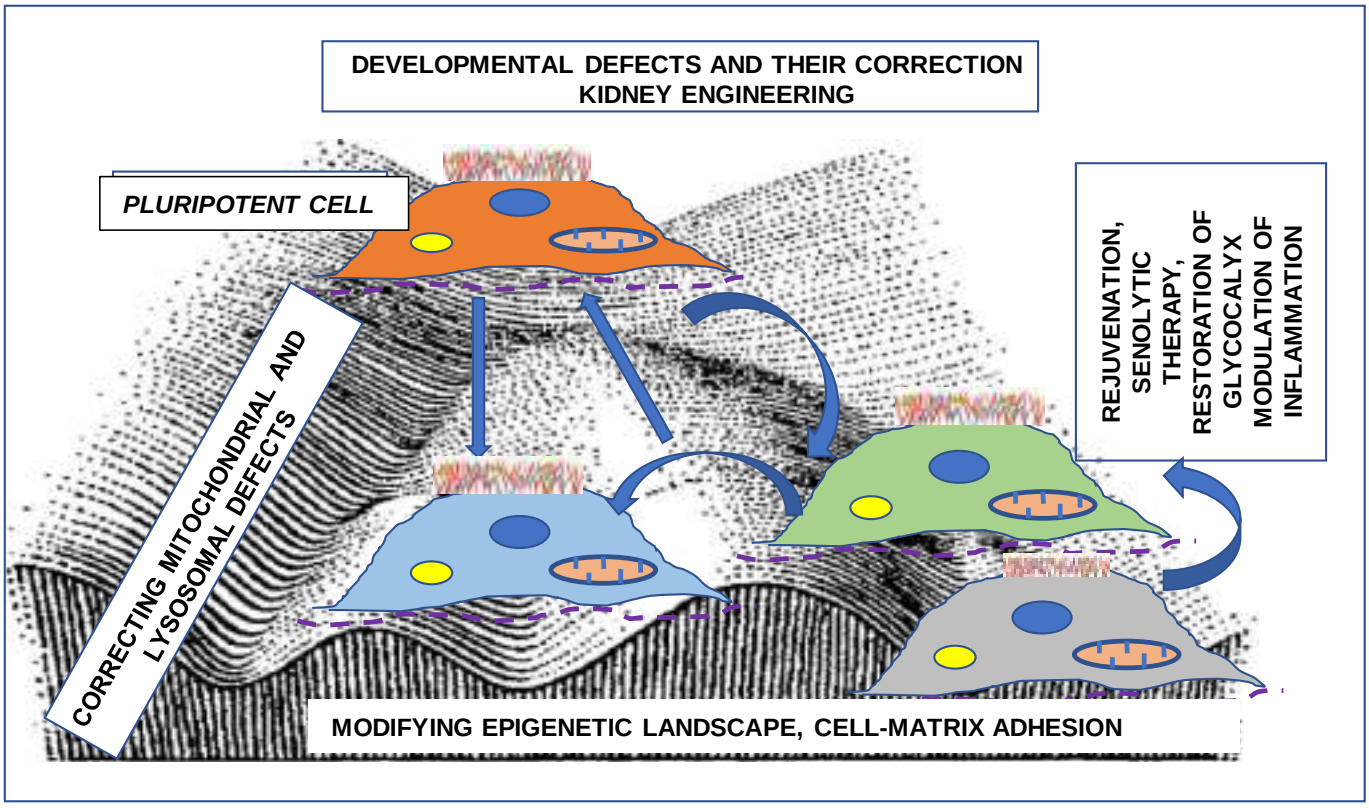
DEVELOPMENTAL DEFECTS AND THEIR CORRECTION
KIDNEY ENGINEERING
PLURIPOTENT CELL
CORRECTING MITOCHONDRIAL AND LYSOSOMAL DEFECTS
REJUVENATION, SENOLYTIC THERAPY, RESTORATION OF GLYCOCALYX MODULATION OF INFLAMMATION
MODIFYING EPIGENETIC LANDSCAPE, CELL-MATRIX ADHESION

FIG. 1 Modified Waddington plot showing diverse pathways that are employed or have future potential for regeneration of the injured kidney. Those include developmental problems and their resolution, correction of organellar defects, resolution of inflammation, engineering of distinct portions of the nephron, cell reprogramming, cell reconditioning, rejuvenation of cellular pool, senolysis, epigenetic and cell-matrix adhesion approaches. *Modified from MS Goligorsky. New trends in regenerative medicine: reprogramming and reconditioning. J Am Soc Nephrol 30;2019:2047–2051.*

In Jorge Luis Borges's story "The Rose of Paracelsus," a potential apprentice insists that the Old Master confirm his mysterious powers by restoring to life a burned rose. Paracelsus "poured the delicate fistful of ashes from one hand into the concave other, and he whispered a single word. The rose appeared again." This short story allegorically embodies the fledgling science of regeneration. Accordingly, the book is expected to serve as an addendum to traditional techniques of life support in patients with failing kidneys, including dialysis and transplantation. In designing the second edition, I tried not to duplicate other similar books that have offered excellent perspectives on developmental subjects and issues related to kidney transplantation.

The gestational period of this book was a difficult one. It has fallen on challenging times. Although it was conceived prior to the SARS-CoV-2 pandemic, all chapters were written at the height of it. While previous experiences with creative work during epidemics—gems like Boccaccio's *Decameron*, Newton's theory of gravity, Pushkin's short stories, are just a few examples—have guided the authors to produce their *capolavoro*, it has been tough for all, especially those who have had mounting clinical responsibilities and/or online tutoring. It has been tantalizingly difficult fighting infection at the frontlines while writing chapters, akin to the work of war correspondents.

In seeing this book to fruition, I am especially grateful to Elizabeth Brown, senior acquisition editor, for encouraging me to produce a second edition of *Regenerative Nephrology*, to editorial assistant Samantha Allard, to production manager Sreejith Viswanathan, and to the entire Elsevier team for navigating it across the rough waters. I also take upon myself full responsibility for the choices of subjects, tentative interpretation of the directions the field of kidney regeneration is heading and, hence, any possible omissions.

Michael S. Goligorsky

PART I

Kidney development and regeneration

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CHAPTER

1

Nephrogenesis in health and disease

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Introduction

The mammalian renal tract is a physically integrated group of organs whose main functions are to generate and void urine [1, 2]. The renal tract comprises the kidney and the urinary tract, the latter incorporating the renal pelvis, the ureter, the urinary bladder, and the urethra. The embryonic origin and subsequent shaping, or morphogenesis, and cellular differentiation of the renal tract are multistep processes, so they are unsurprisingly prone to error. This chapter addresses the normal development of the renal tract, with an emphasis on the early steps of development of the metanephric kidney. We then proceed to consider some genetic and environmental causes of renal tract malformations. Given that the theme of this book is regeneration, we consider whether aberrations of renal tract development can be rescued by manipulation of, for example, growth factor signaling.

Experimental models

The mechanisms of mammalian renal tract development have been most extensively studied in rodents, although the anatomy of human renal tract development has been described in detail by Edith Potter over 50 years ago [3]. The anatomical steps of renal tract development are similar in mice, rats, and humans, although the timetable is accelerated in the rodents. There are also differences of scale between humans and rodents. For example, the healthy human kidney contains around 1.4 million glomeruli [4], while a healthy mouse [5] or rat [6] kidney respectively contain around 6 and 30 thousand glomeruli. Our focus is mammalian development but readers who are interested in how kidneys form in frogs, fish, and flies are directed to comprehensive reviews published elsewhere [1]. Although covered in detail in other chapters, we briefly allude to pluripotent stem cell technology which is increasingly being used to study kidney development, complementing the study of native renal tracts.

Developmental events preceding kidney development

The primitive streak arises toward the posterior end of the back of the early embryo. The streak coordinates the migration of cells from the surface of the embryo such that they become the three germ layers: the endoderm, mesoderm, and ectoderm. These gastrulation events are driven by gradients of growth factors such as wingless-related integration site-3A (WNT3A) and bone morphogenetic protein 4 (BMP4) [7, 8]. The portion of mesoderm called the intermediate mesoderm will give rise to the embryonic kidneys, of which there are three sets of paired organs. The pronephric and mesonephric kidneys form but then regress in the embryonic period and they are not discussed

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further here. In contrast, the metanephric kidney, or metanephros, forms and survives to become the definitive mammalian kidney. It initiates on day 10 of mouse gestation, day 12 of rat gestation, and week 5 of human gestation.

Cell populations in the metanephric kidney

The metanephros contains two populations of precursor cells, the ureteric bud and the metanephric mesenchyme, as depicted in Fig. 1.1 [9]. Based on mouse experiments [10], ureteric bud precursor cells express the transcription factor called odd-skipped-related 1 (OSR1). They are more anteriorly positioned within the intermediate mesoderm than the precursor cells that will become metanephric mesenchyme; the latter express the T-box transcription factor T (TBXT, or brachyury). The ureteric bud is an epithelial tube that branches from the caudal part of the Wolffian duct of which there are two, each formed from the intermediate mesoderm and extending caudally toward the cloaca, the precursor of the urinary bladder and hindgut [11]. The stalk of the ureteric bud will give rise to the urothelium of the ureter, while its top will branch serially to form the collecting ducts of the metanephric kidney. The first sets of branches are remodeled to become the epithelia of the renal pelvis [2]. Therefore, all these derived cells are said to be in the "ureteric bud lineage." The metanephric mesenchyme will form the nephrons (i.e., podocytes, proximal tubule, loop of Henle, and distal convoluted tubule), and additionally contributes to kidney interstitial cells. Moreover, a subset of cells in the metanephric mesenchyme express markers of endothelial precursors and, at least experimentally, can contribute to kidney interstitial and glomerular capillaries [12]. Fig. 1.2 shows histology of three time points from the rat metanephros where early ureteric bud branch tips and primitive nephrons are evident.

Molecular control of kidney development: The ureteric bud/collecting duct lineage

As reviewed [13], the correct point of emergence of the ureteric bud is controlled by a balance of stimulatory and inhibitory growth factors. For example, BMP4, secreted by mesoderm that is not fated to become metanephric mesenchyme, inhibits emergence, whereas glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 10 (FGF10), both secreted by the definitive metanephric mesenchyme, stimulate bud emergence. Within the domain of the Wolffian duct where the bud will emerge, cells upregulate rearranged during transfection (RET), the receptor tyrosine kinase that binds GDNF [14]. The Fraser extracellular matrix complex subunit 1 (FRAS1) protein, and family members FRAS1-related extracellular matrix 1 (FREM1) and FREM2, together coat the surface of the ureteric bud and are required to optimize GNDF signaling [15, 16]. The FRAS1/FREM1/FREM2 complex also facilitates the binding of the matrix molecule nephronectin to integrin α8β1 that is required for physical interaction between the ureteric bud and metanephric mesenchyme [17]. Fine-tuning of branching is mediated by an intrinsic ureteric

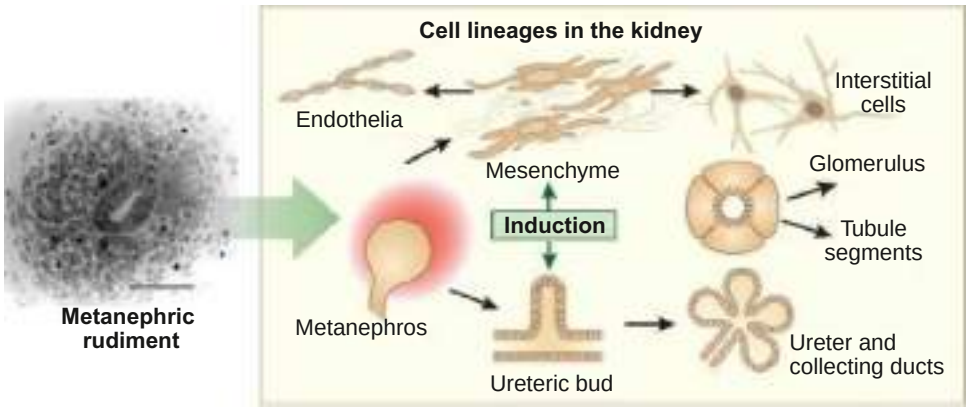

FIG. 1.1 Cell lineages in the embryonic metanephros. The frame on the left shows the histology of the metanephros at its inception, with a central ureteric bud surrounded by metanephric mesenchyme. Bar = 50 μm. The frame on the right depicts mutual induction between these compartments. The ureteric bud differentiates into the urothelial stalk of the ureter and the arborizing collecting ducts within the kidney. The metanephric mesenchyme undergoes mesenchymal to epithelial transition to form nephrons, comprising glomerular and tubule epithelia, whereas other cells in the mesenchymal compartment will form interstitial cells and endothelia. *Reproduced from Woolf AS. Growing a new human kidney. Kidney Int 2019;96:871–882.*

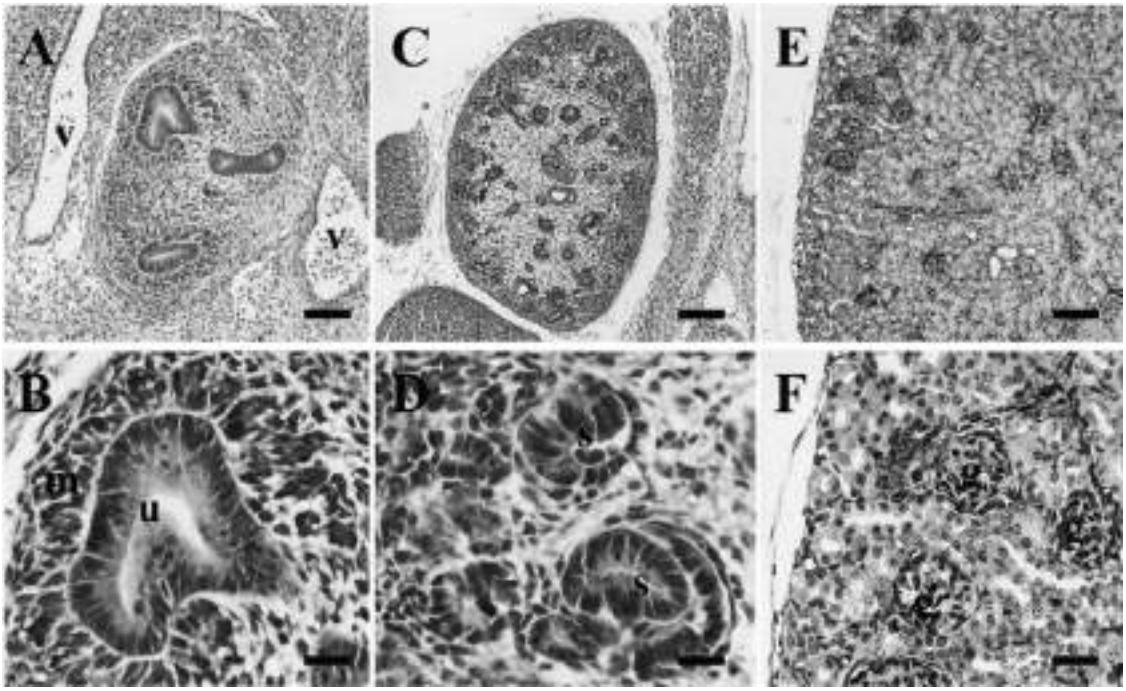

FIG. 1.2 Bright field images of maturation of the embryonic kidney of the rat. Sections from control animals were stained with hematoxylin and eosin. (A and B) Embryonic day 13 metanephros with ureteric bud branch tips (u) and condensing mesenchyme (m). Nearby vessels are indicated (v). (C and D) Embryonic day 15 metanephros shows considerable growth and differentiation but the most mature nephron stages are S-shaped bodies (s) and an outer nephrogenic zone is still prominent. (E and F) Two-week postnatal kidney. Note that there is no nephrogenic zone and that all glomeruli (g) appear mature with capillary loops. Bars are: 100 µm in A, C and E, and 10 µm in B, D and F. *Reproduced from Welham SJM, Wade A, Woolf AS. Protein restriction in pregnancy is associated with increased apoptosis of mesenchymal cells at the start of rat metanephrogenesis. Kidney Int 2002;61:1231–1242.*

bud molecule called sprouty-1 which antagonizes GDNF and FGF10 signaling. Sprouty-1 itself is downregulated by angiotensin II [18], and BMP4 is antagonized by yet another secreted factor, gremlin-1 [19]. Serial branching of the ureteric bud forms the collecting duct tree, a process again primarily driven by GDNF, secreted by the cortical rim of undifferentiated mesenchyme. The formation of the tree is fine-tuned by numerous other molecules including those encoded by the neurofibromatosis 2 gene *NF2* [20] and the planar cell polarity genes called Van Gogh-like protein 2 (*VANGL2*) and cadherin EGF LAG seven-pass G-type receptor 1 (*CELSR1*) (Fig. 1.3) [21].

Molecular control of kidney development: The metanephric mesenchyme/nephron lineage

Metanephric mesenchymal cells express the Wilms tumor 1 (WT1) transcription factor, without which they regress prematurely [22], another aberrant pathway to renal agenesis. In health, cells in this compartment undergo rounds of mesenchymal-to-epithelial transition to form multiple layers of nephrons (Fig. 1.2), the deepest layers having formed first. In mice, the waves of nephron formation, literally "nephrogenesis," continue until a week after birth, whereas in humans the process is complete by 34 weeks of gestation. At the same time as layers of nephrons are being generated, the expression of FGF9 and FGF20 growth factors and the sine oculis homeobox homolog 2 (SIX2) transcription factor preserve a rim of metanephric mesenchyme that will give rise to further rounds of nephrons; without these growth molecules, there is premature exhaustion of nephron progenitors [23, 24]. During the development of the metanephric kidney, subsets of cells have a high proliferative rate, especially those in the branching tips of the ureteric tree, and in primitive nephrons [25]. Concurrently, apoptctic death takes place in the developing metanephros, and it has been estimated that around half the cells generated in the metanephros will undergo programmed cell death during development [26]. Apoptosis is prominent in cells around emerging nephrons, as depicted in Fig. 1.4 [27]. Here, perhaps interstitial cells need to be deleted to facilitate sculpting of the nephron. Apoptosis is also prominent in the deep medulla, perhaps to make way for loops of Henle that grow into the forming papilla. Indeed, if apoptosis is experimentally inhibited in metanephric organ culture, then nephrogenesis is perturbed [28].

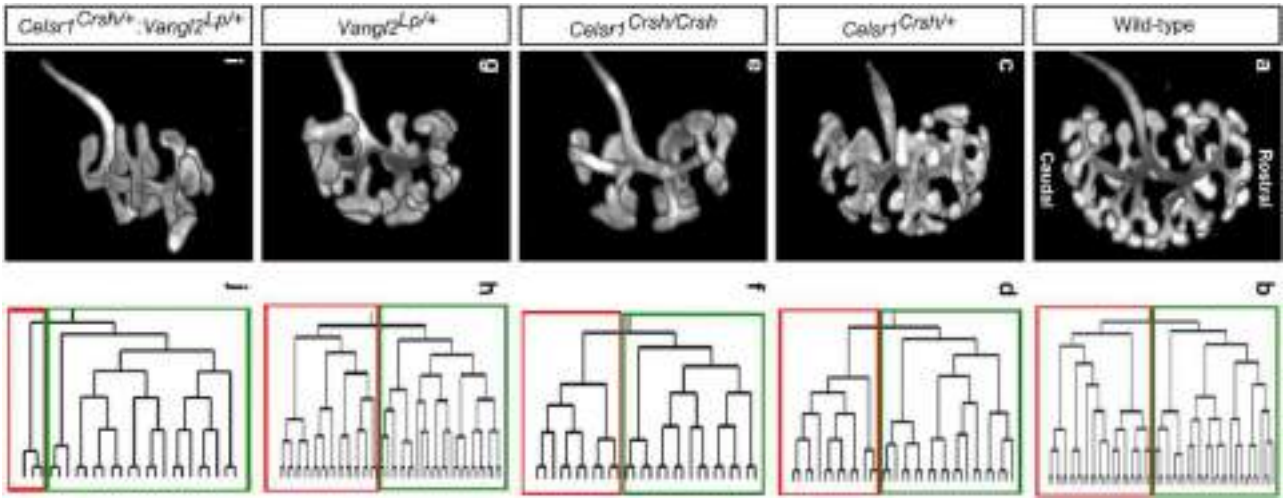

FIG. 1.3 Patterns of ureteric bud branching in mice with mutations in planar cell polarity genes. Images of whole metanephroi from embryonic day 13 wild-type (A), *Celsr1*$^{Crsh/+}$ (C), *Celsr1*$^{Crsh/Crsh}$ (E), *Vangl2*$^{Lp/+}$ (G), and *Celsr1*$^{Crsh/+}$:*Vangl2*$^{Lp/+}$ (I) mice stained with calbindin-D28K and visualized by optical projection tomography. Branching networks of individual metanephroi from embryonic day wild-type (B), *Celsr1*$^{Crsh/+}$ (D), *Celsr1*$^{Crsh/Crsh}$ (F), *Vangl2*$^{Lp/+}$ (H), and *Celsr1*$^{Crsh/+}$:*Vangl2*$^{Lp/+}$ (J) mice. Note the impaired branching patterns in the homozygous mutant and compound heterozygous mutant embryonic kidneys. *Reproduced from Brzóska HL, d'Esposito AM, Kolatsi-Joannou M, Patel V, Igarashi P, Lei Y, et al. Planar cell polarity genes Celsr1 and Vangl2 are necessary for mammalian kidney growth, differentiation and rostrocaudal patterning. Kidney Int 2016;90:1274–1284.*

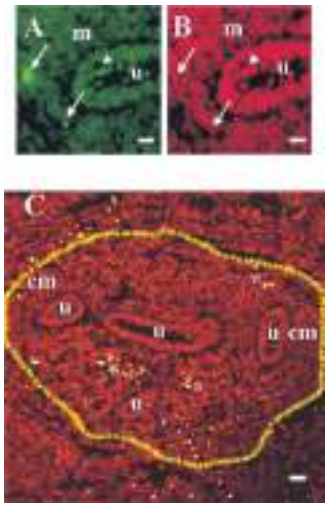

FIG. 1.4 Confocal laser scanning images of the embryonic day 13 rat metanephros. Sections from control diets. (A and B) The same area under appropriate wavelengths for detection of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and propidium iodide-labeled nuclei, respectively. Two TUNEL-labeled nuclei (*bright green*) associated with condensing mesenchyme are indicated in panel A (*arrows*) and appear as pyknotic nuclei stained red with propidium iodide (*arrows* in panel B). A ureteric bud branch (u) and mesenchyme (m) are indicated. The *arrowhead* indicates a mitotic cell. (C) Whole embryonic day metanephros, outlined for clarity by a *dotted line*, and surrounding tissues. The photograph was generated by merging the signals from both wavelengths, with apoptotic TUNEL/propidium iodide-stained nuclei appearing *bright yellow*. Note the apoptotic cells, often in clusters in loose mesenchyme (*). Apoptosis was also seen, but less prominently, in condensed mesenchyme (cm) and was not detected in ureteric bud branches (u). Bars are 10 μm in A and B, and 30 μm in C. *Reproduced from Welham SJM, Wade A, Woolf AS. Protein restriction in pregnancy is associated with increased apoptosis of mesenchymal cells at the start of rat metanephrogenesis. Kidney Int 2002;61:1231–1242.*

The morphological journey to become a nephron begins close to a ureteric bud branch tip, or ampulla, where a subset of cells in the metanephric mesenchyme are induced to aggregate and then form a vesicle, essentially an epithelial sphere. The vesicle elongates and curves via stages called the comma and S-shaped body. During elongation, the maturing nephron patterns, proximally–distally, into glomerular podocytes, the proximal tubule, the loop of Henle, and the distal convoluted tubule. The latter fuses with a collecting duct to form a patent conduit. WNT4 is a key molecule that drives the generation of primitive nephron vesicles from mesenchymal precursors [29].

The transmembrane molecule called neurogenic locus notch homolog protein 2 (NOTCH2) is required for differentiation of the proximal segment of the primitive nephron segments (i.e., podocytes and the proximal tubule) [30]. These proximal segment fates are also controlled by hepatocyte nuclear factor-1B (HNF1B) [31], a transcription factor that also has roles in controlling mitochondrial respiration in kidney tubules [32] and enhancing the differentiation of collecting duct epithelia [33].

Glomerular podocytes differentiate from cuboidal into mature flattened epithelia that extend foot processes from their basal surface which abut on the glomerular basement membrane [34]. The maturing podocytes upregulate WT1 to levels higher than that found in metanephric mesenchyme [25]. This is accompanied by the expression of genes that code for slit diaphragm proteins, including nephrin and podocin. At the same time, the composition of the glomerular basement membrane matures to include collagen IV α3, α4, and α5, as well as laminin B2. Of note, this protein maturation fails to take place in organ culture, a scenario where the glomerular tuft lacks capillary loops that are found in vivo. In native developing kidneys, maturing podocytes secrete vascular growth factor A and platelet-derived growth factor B, and gradients of these molecules attract precursor cells to migrate into the tuft and respectively form capillary loops [35] and mesangial cells [36]. Of note, vascularized glomeruli expressing basement membrane can be generated after subcutaneous implantation of human pluripotent stem cell-derived kidney precursor cells into immunocompromised mice [37], as depicted in Fig. 1.5.

Urinary tract development

The urinary tract comprises the renal pelvis, the ureter, the urinary bladder, and the urethra. The ureters propel urine from the renal pelvis to the bladder. In turn, the bladder stores urine at low pressure until the detrusor smooth muscle in the bladder wall contracts, resulting in complete voiding of its contents through the urethra. The mammalian ureter, a wholly mesodermal-derived organ, initiates when the ureteric bud branches from the Wolffian duct. The epithelial bud then elongates to form the stalk of the ureter, at the same time differentiating to form the pseudostratified urothelium of the ureter and renal pelvis [38]. The urothelium expresses uroplakin proteins that confer a water-tight barrier, preventing the egress of urine out of the urinary tract [39]. Concurrently, mesenchyme condenses around the urothelial tube and differentiates into a smooth muscle [40]. The urothelial layer of the bladder differentiates from the endoderm-derived urogenital sinus, and adjacent mesoderm-derived mesenchymal cells differentiate into smooth muscle. The embryonic urothelium acts as a signaling center [41, 42], secreting sonic hedgehog (SHH) that acts on nearby mesenchymal cells to drive their proliferation and differentiation into smooth muscle. This involves the muscle precursor cells secreting BMP4 that leads to the transcription factors teashirt-3 and myocardin upregulating the expression of smooth muscle contractile proteins such as smooth muscle actin and myosin heavy chain [40, 43]. The caudal end of the Wolffian duct fuses with the urogenital sinus, facilitated by RET [44], and then involutes through vitamin A-mediated apoptosis [45]; the result is that the distal end of the forming

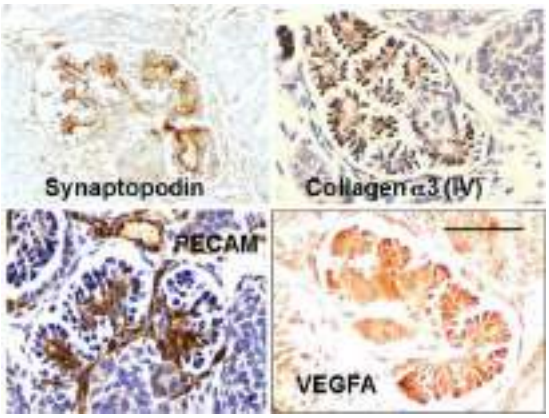

FIG. 1.5 Mature glomeruli formed in vivo after implanting human pluripotent stem cell-derived kidney precursor cells into immunocompromised mice. Glomerular tufts immunostain (*brown*) for the podocyte marker synaptopodin. Glomeruli immunostain for collagen α3 (IV) and vascular endothelial growth factor A (VEGFA). Capillaries that immunostain for platelet endothelial cell adhesion molecule (PECAM) are detected inside the glomerular tuft. Nuclei in the collagen α3 (IV) and PECAM frames are counterstained with hematoxylin. Bar = 50 μm. *Reproduced from Woolf AS. Growing a new human kidney. Kidney Int 2019;96:871–882; Adapted from Bantounas I, Ranjzad P, Tengku F, Silajdžić E, Forster D, Asselin MC, et al. Generation of functioning nephrons by implanting human pluripotent stem cell-derived kidney progenitors. Stem Cell Reports 2018;10:766–779.*

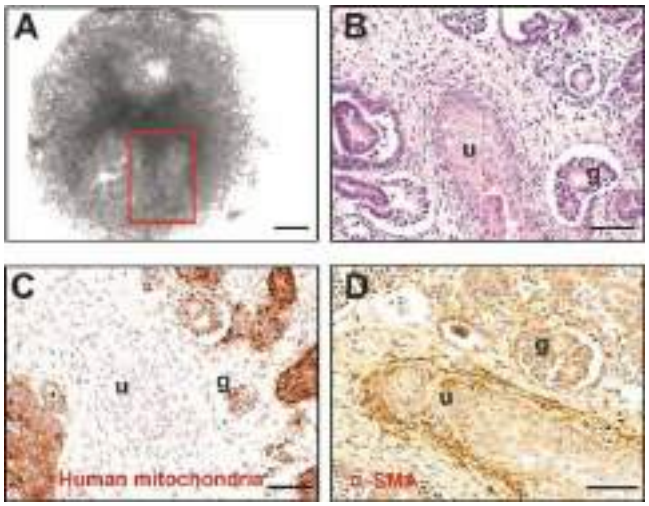

FIG. 1.6 A chimeric organoid containing nephrons derived from human pluripotent stem cells and an embryonic mouse ureter. (A) The organoid visualized in culture with the mouse ureter (the vertical tube inside the red box) surrounded by human nephron-like tissues. (B) Histology of the boxed zone shows the mouse ureter (u) flanked by glomeruli (g) in this section stained with hematoxylin and eosin. (C) Nearby section immunostained with an antibody to human mitochondria (*brown positive signal*) confirms that the glomeruli are of human origin. (D) The embryonic mouse ureter has differentiated to form an α-smooth muscle actin positive (α-SMA) coat (*brown*), as it does in vivo. Bar in A is 500 μm, and bars in B–D are 50 μm. *Adapted from Ashley S. Plumbing kidney organoids derived from human pluripotent stem cells. M Res Thesis, University of Manchester. 2019, pp. 1–100.*

ureter becomes incorporated into the lateral aspect of the bladder where the uretero-vesical junction acts as a valve that prevents reflux of urine. The bladder is richly innervated [46] and, in humans, from the late first trimester autonomic nerves are visualized, extending between smooth muscle bundles [47]. These postganglionic neurons emanate from autonomic ganglia outside the bladder and they express heparanase 2 (HPSE2) and leucine-rich repeats and immunoglobulin-like domains protein 2 (LRIG2) proteins that appear to pattern their distribution [48]. Urinary tract differentiation harmonizes with kidney development, so that the tract is ready to receive and transmit urine made by the developing kidney. Indeed by the end of the first trimester of human gestation, the bladder is a muscular organ that receives and voids urine.

As yet, protocols have not been published that describe the simultaneous generation of both a kidney and ureter from pluripotent stem cells. However, such stem cells can be driven toward a urothelial fate and, when recombined with native mouse intermediate mesoderm from near the Wolffian duct, a ureter-like contractile tube can be formed [49].

As depicted in Fig. 1.6, a chimeric renal organoid containing nephrons derived from human pluripotent stem cells enveloping an embryonic mouse ureter can be generated by tissue engineering [50].

Abnormal development of the renal tract

The embryonic origin and subsequent shaping, or morphogenesis, and cellular differentiation of the renal tract are multistep processes, so they are unsurprisingly prone to error. Renal tract malformations, sometimes also called Congenital Anomalies of the Kidney and Urinary Tract (CAKUT), are a collection of rare disorders, yet this disease category is the primary diagnosis in half of all children with end-stage renal disease (ESRD) [51]. Such malformations also account for up to a fifth of young adults with ESRD [52]. Moreover, a third of all anatomical defects detected by prenatal ultrasound scan screening are renal tract malformations, and the most severely affected fetuses often undergo elective termination of pregnancy [53]. Increasingly, human renal tract malformations are being found to have genetic bases [54, 55], although it is likely that environmental perturbations also can occur.

Models of renal agenesis

The emergence of the ureteric bud from the Wolffian duct can be considered as the critical event in the formation of the metanephric kidney. Indeed, if the bud does not initiate and grow into the metanephric mesenchyme, the result is an absent kidney and ureter, or renal agenesis. Renal tract development will also be compromised if the bud

emerges either too high (cranial) or too low (caudal) along the Wolffian duct. These respective scenarios will result in a malformed (dysplastic) kidney or a ureter with a too lateral insertion into the bladder. A third possible aberration is the emergence of multiple ureteric buds, leading to a duplicated (duplex) renal tract. An example of renal agenesis is provided by Fraser syndrome where individuals carry biallelic mutations of either *FRAS1* or *FREM2* and often lack both kidneys [15, 16]. As discussed earlier, the encoded proteins coat the surface of the ureteric bud and facilitate paracrine signaling by mesenchymal-derived growth factors. Remarkably, this most severe of all renal tract malformation is not "written in stone," at least in an experimental mouse model. Examination of *Fras1* homozygous mutant mice shows that the ureteric bud either fails to initiate or, if it does, it fails to fully engage with the metanephric mesenchyme. The result in vivo is that the mesenchyme dies and later in gestation kidney tissue cannot be found. If the zone comprising the mutant caudal Wolffian duct and surrounding intermediate mesoderm is explanted in organ culture, a similar sequence of events occurs [15]. If, however, the culture is supplemented with high concentrations of GDNF or FGF10, the metanephric development proceeds such that a small rudiment is formed. Strikingly, if the homozygous *Fras1* null mutation is placed on a genetic background where sprouty-1 expression is less than normal, kidney development proceeds so that two kidneys are generated that can sustain the life of *Fras1* mutant through to adulthood [16].

There are also environmental causes of renal agenesis. For example, overexposure of developing embryonic mice, before the kidney initiates, to retinoic acid, a metabolite of vitamin A, leads to the downregulation of WT1 in metanephric mesenchyme which proceeds to degenerate with upregulated apoptosis [56]. Again, as for the *Fras1* genetic example above, this sequence leading to agenesis can be mimicked *in vitro* when rudiments, harvested from retinoic acid exposed mice, are explanted in organ culture (Fig. 1.7). Notably, the development of these rudiments can be

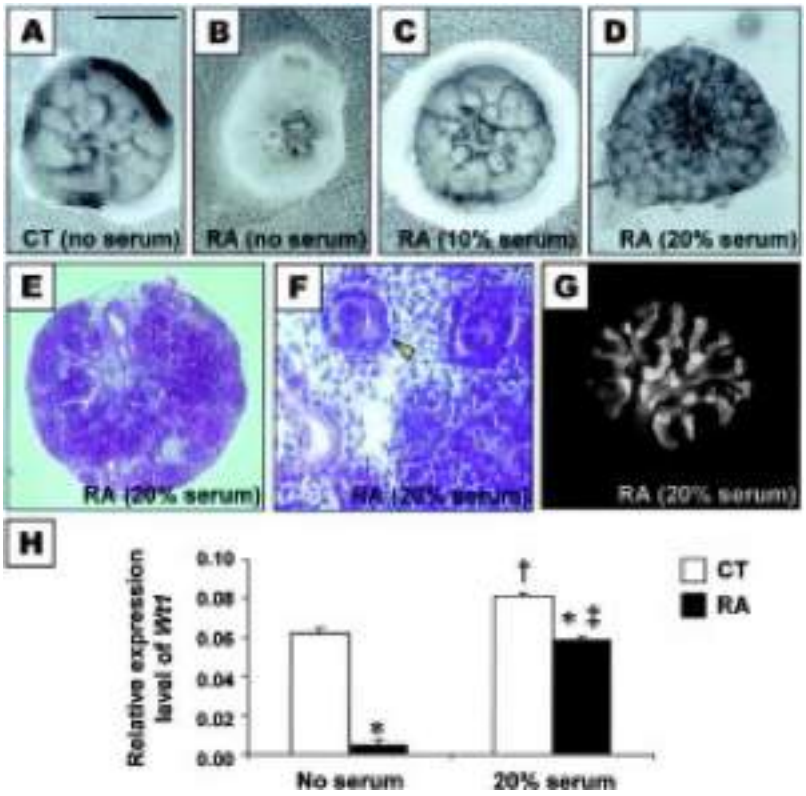

FIG. 1.7 Mouse metanephric explants cultured in vitro for 5 days. (A–D) Morphology of explanted metanephroi from control (CT) or retinoic acid-treated (RA) embryos cultured in serum-free medium (A and B), or medium supplemented with 10% (C) or 20% serum (D). E and F. Histology of RA-treated metanephric explants cultured in 20% serum showed that the rudiments had undergone differentiation, with formation of condensates, primitive tubules, and avascular glomeruli (*arrowhead*). (G) Calbindin immunostaining of UB branch epithelium showed multiple branching in metanephric explants from RA-treated embryos after culture in 20% serum. (H) Real-time quantitative RT-PCR analyses of expression levels of *Wt1* relative to β-*actin* in metanephroi from control and RA-exposed embryos at 2 days after culture in serum-free medium or medium supplemented with 20% serum [*, $P < 0.0001$, CT versus RA in the same culture condition; †, $P < 0.005$, CT (no serum) versus CT (20% serum); ‡, $P < 0.0001$, RA (no serum) versus RA (20% serum); Student's *t*-test]. Scale bar: 0.5 mm (A–D, G), 0.35 mm (E), 1.4 mm (F). *Reproduced from Tse HK, Leung MB, Woolf AS, Menke AL, Hastie ND, Gosling JA, et al. Implication of Wt1 in the pathogenesis of nephrogenic failure in a mouse model of retinoic acid-induced caudal regression syndrome. Am J Pathol 2005;166:1295–1307.*

rescued by the addition of high concentrations of serum [56]. Perhaps a yet-to-be defined growth factor present in serum mediates this effect, and it has been shown that the addition of epidermal growth factor can minimize apoptosis in otherwise normal embryonic rodent kidneys [26].

Models of renal dysplasia

Another phenotype of human kidney maldevelopment is renal dysplasia, in which the kidney begins to form but its differentiation is incomplete, with impaired branching morphogenesis and nephrogenesis, and metaplastic, for example, with the generation of smooth muscle and cartilage (Fig. 1.8). As for renal agenesis, there are genetic and environmental causes. An example of the former are heterozygous mutations of *HNF1B*, currently the commonest genetically defined cause of human renal dysplasia [54, 57, 58]. As discussed earlier, this gene codes for a transcription factor with multiple roles in morphogenesis and differentiation of epithelia in both the ureteric bud and metanephric mesenchymal lineages. Human renal dysplastic kidneys have been shown to display a significantly increased number of apoptotic cells compared with time-matched healthy control kidneys [59], perhaps explaining the tendency of these organs to regress in size either before birth or in the first years of childhood.

Renal dysplasia, however, is also frequently observed in association with fetal urinary flow impairment [43]. Indeed, impaired nephrogenesis can be experimentally generated by, for example, ligating the ureter of a fetal sheep [60], as depicted in Fig. 1.9. Similar disruption of nephrogenesis follows experimental ligation of the fetal urethra [61]. This physical insult triggers upregulation of transforming growth factor β (TGFβ) in the kidney [60]. Moreover, the addition of this factor to an epithelial cell line derived from a human dysplastic kidney leads to transdifferentiation to a smooth-muscle-like phenotype, as shown in Fig. 1.10 [62]. The addition of TGFβ to mouse embryonic ureteric explants retards their linear growth; this is associated with a tendency for downregulation of transcripts encoding FGF10, and exogenous FGF10 can partially rescue the linear growth defect in TGFβ-exposed ureteric explants [38].

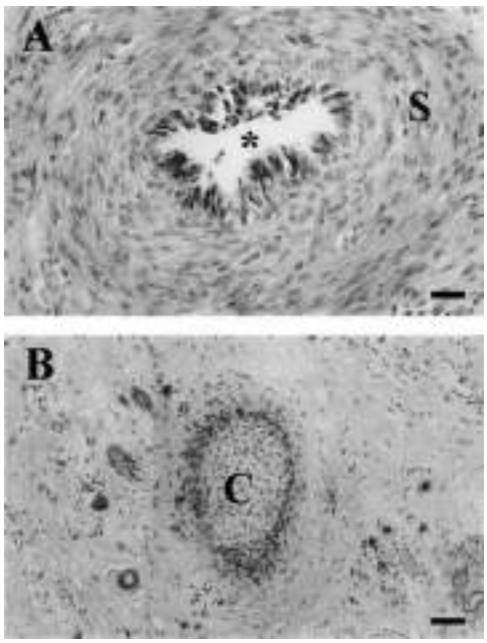

FIG. 1.8 The histology of human renal dysplasia stained with hematoxylin and eosin. (A) Dysplastic tubule (*marks the lumen) surrounded by poorly differentiated stromal tissues(s). (B) Metaplastic cartilage (c). Note the absence of normal nephrons and glomeruli. Bars are 15 μ in A and 150 μ in B. *Reproduced from Bingham C, Ellard S, Cole TR, Jones KE, Allen LI, Goodship JA, et al. Solitary functioning kidney and diverse genital tract malformations associated with HNF1b mutations. Kidney Int 2002;61:1243–1251.*

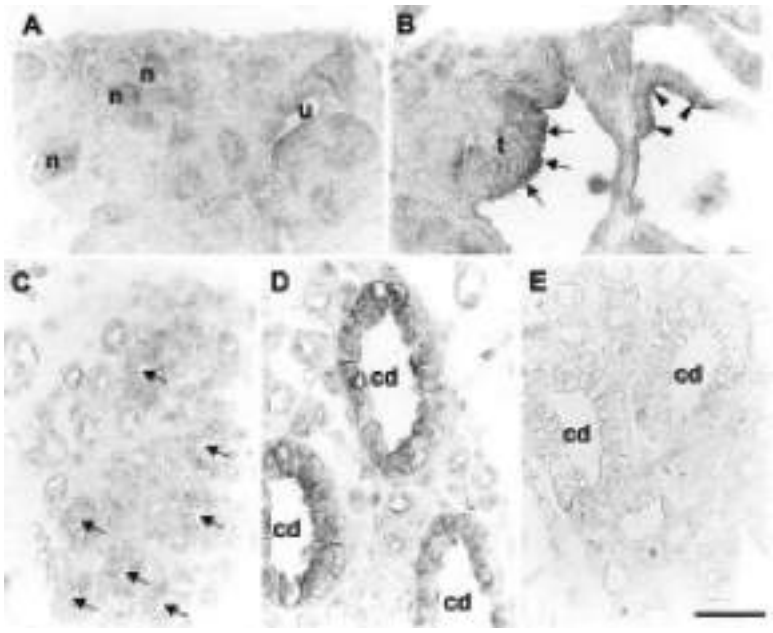

FIG. 1.9 Ureteric ligation leads to impaired kidney development and upregulation of *TGFB1* expression. *TGFβ1* in situ hybridization in sham-operated and obstructed sheep fetal kidneys. A and C are sections of sham-operated developing kidneys, whereas B, D, and E are from obstructed kidneys. All panels represent the results of hybridization with *TGFβ1* antisense probes, apart from E in which a control sense probe was used. (A) *TGFβ1* transcripts were detected in ureteric bud (u) and forming nephrons (n) in the normal superficial cortex. (B) Prominent signal for *TGFβ1* was observed in dilated tubule epithelia (*arrowheads*) and glomerular tufts (t;*arrows*) in the obstructed cortex. (C) Faint *TGFβ1* signal was detected in sham-operated developing medullary collecting ducts (*arrows*). (D) In contrast, there was TGF-β1 transcript up-regulation in the larger dilated collecting ducts (cd) in the obstructed kidneys. (E) Only background signal was detected using the sense probe. Scale bar, 15 μm. *Reproduced from Yang SP, Woolf AS, Quinn F, Winyard PJD. Deregulation of renal transforming growth factor-β1 after experimental short-term ureteric obstruction in fetal sheep. Am J Pathol 2001;159:109–117.*

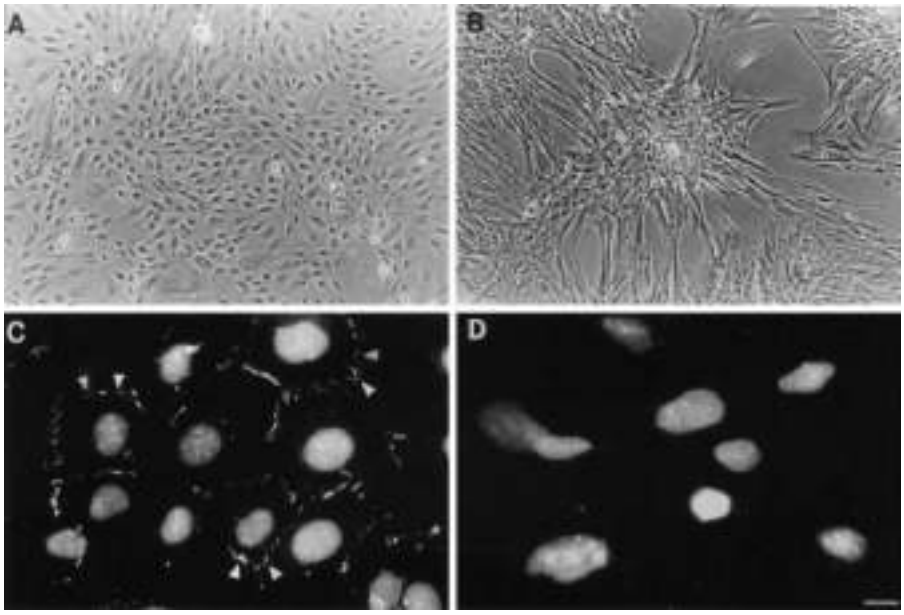

FIG. 1.10 Effects of TGFβ1 on morphology and tight junctions in an epithelial cell line derived from a human dysplastic kidney. Gross morphology is shown in A and B, whereas C and D show tight junction zonula occludens-1 (ZO1) immunocytochemistry (with propidium-iodide nuclear counterstaining) of epithelial-like dysplastic cells cultured in either control medium (A and C) or with exogenous TGF-β1 (B and D). (A) Cells cultured in control medium had an epithelial-like morphology in monolayer culture. (B) Morphological changes were observed after exposure to 2.0 ng/mL TGFβ1 for 72 h: multilayered aggregates formed in semiconfluent and confluent cultures and individual cells between aggregates became larger and developed filopodia and lamellipodia characteristic of a motile phenotype. (C and D) In control medium ZO1 was immunolocalized (*white speckled lines*) to lateral cell junctions (*arrowheads*). But immunostaining at cell borders was lost after culture with TGFβ1. Scale bar: 40 μm (A and B); 15 μm (C and D). *Reproduced from Yang SP, Woolf AS, Yuan HT, Scott RJ, Risdon RA, O'Hare MJ, Winyard PJD. Potential biological role of transforming growth factor β1 in human congenital kidney malformations. Am J Pathol 2000;157:1633-1647.*

Models of renal hypoplasia

The mildest type of kidney malformation is renal hypoplasia, where the kidney contains normally differentiated tissues but there are fewer nephrons than normal. Of note, even in overtly healthy, normotensive, humans there exists a wide range of nephron numbers, as assessed by counting glomeruli; the range is remarkable, from around half a million to two million [4]. Notably, individuals with a history of essential hypertension have, on average, half as many glomeruli per kidney [4]. Again, we can invoke genetic and environmental causes to explain variation in nephron number. For example, as shown in Fig. 1.11, glomerular numbers vary significantly between different strains of "wild-type" mice, and this may be attributed to yet-to-be defined genetic variation, e.g., C57BL6 has more glomeruli than FVB mice [5]. Moreover, within each strain, females tend to have more glomeruli than males [5], and one could hypothesize that testosterone inhibits nephrogenesis. In these studies, it was noted that the level of urinary albumin excretion in adult mice was inversely correlated with glomerular number [5], as shown in Fig. 1.12.

A well-established example of an environmental perturbation able to generate renal hypoplasia is low protein diet initiated from the start of gestation in pregnant rats. Here, the offspring have significantly fewer glomeruli than normal. Moreover, this manipulation causes a markedly increased prevalence of apoptosis in the just-formed metanephros with a subsequent depletion in cell numbers [6]. Moreover, the low protein diet is associated with altered gene expression in the developing kidney [6]; the genes include those coding for PROX1, the ortholog of Drosophila transcription factor prospero, and cofilin-1, a regulator of the actin cytoskeleton. It has been reasoned that the effects of maternal low protein diet are in part mediated by downregulation of placental 11β-hydroxysteroid dehydrogenase-2 (HSD11B2), an enzyme degrading maternal corticosteroids [63]; this would lead to the embryo being exposed to high levels of glucocorticoids. Explants of mouse metanephric kidneys treated with dexamethasone, a glucocorticoid, show impaired overall organ growth as well as cystic change in the metanephric tubules [64]. This is associated with the upregulation of Indian hedgehog, a member of the SHH family, and addition of cyclopamine, a chemical that blocks HH signaling, ameliorates the malformation caused by dexamethasone [64]. An in vivo example of the plasticity in nephron numbers is provided by an experiment in which fetal sheep underwent uninephrectomy. Several weeks later, the remaining kidney had generated significantly more glomeruli than normal [65], perhaps in response to a yet-to-be defined circulating nephrogenic factor.

Urinary tract malformations

Probably, less of understood about the causes of urinary tract malformations, although in the last decade several genes have been found to be mutated in certain individuals with ureter or bladder anomalies. These genes code for transcription factors, smooth muscle contractile proteins, and neurogenic molecules, and the interested reader is directed elsewhere for a review of this topic [55]. As with the kidney, perhaps there are prospects for a degree of plasticity in urinary tract malformations. As an example, in a *DNA (cytosine-5)-methyltransferase 1* mutant mouse model with a primary defect in bladder urothelial differentiation, a population of cells from the Wolffian duct migrate to the bladder and transdifferentiate to urothelium [66].

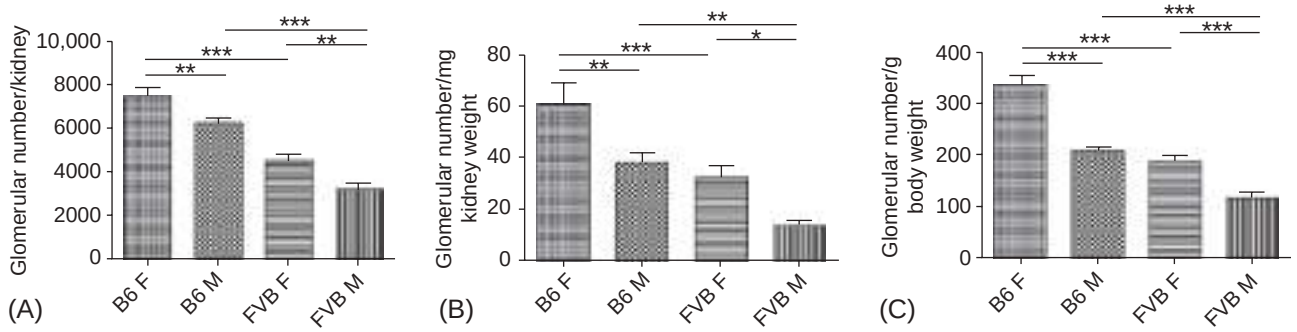

FIG. 1.11 Glomerular counts in C57BL6 and FVB/N 18-week-old mice. (A) A reduction in glomerular number was observed in male (M) mice compared with strain-matched females (F); in addition, FVB/N had significantly lower number of glomeruli compared with sex-matched B6 mice. Similar changes were calculated when either (B) kidney or (C) body weight of the mice was taken into account (*$P<0.05$, **$P<0.01$; ***$P<0.001$ between groups, $n=6$ in each group). All data are presented as means and standard error of the mean. *Reproduced from Long DA, Kolatsi-Joannou M, Price KL, Dessapt-Baradez C, Papakrivopoulou E, Hubank M, Korstanje R, Gnudi L, Woolf AS. Albuminuria is associated with too few glomeruli and too much testosterone. Kidney Int 2013;83:1118–1129.*

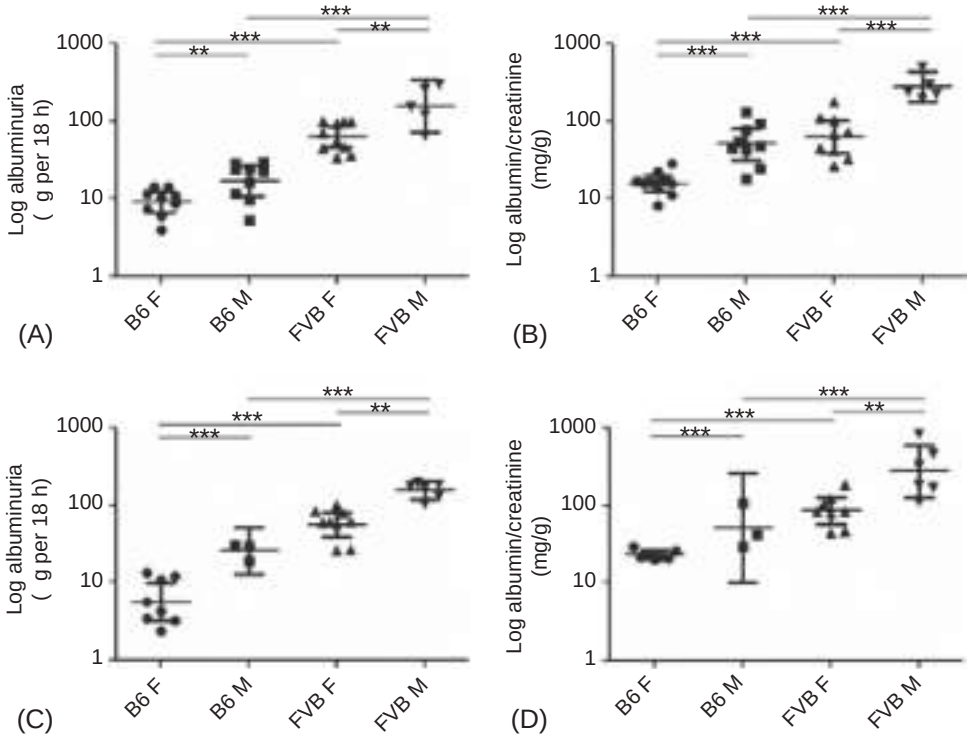

FIG. 1.12 Albuminuria in C57BL6 and FVB/N mice. Overnight (A, C) albumin excretion and (B, D) albumin-to-creatinine ratios were evaluated in (A, B) 18-week-old and (C, D) 13-week-old adult mice. Data were log transformed before analysis and are presented as geometric means and confidence interval. There was a significant increase in urinary albumin in male (M) and female (F) FVB/N mice compared with sex-matched B6 animals. Within each strain, males had elevated albuminuria compared with females (**$P < 0.01$, ***$P < 0.001$ between groups). *Reproduced from Long DA, Kolatsi-Joannou M, Price KL, Dessapt-Baradez C, Papakrivopoulou E, Hubank M, Korstanje R, Gnudi L, Woolf AS. Albuminuria is associated with too few glomeruli and too much testosterone. Kidney Int 2013;83:1118–1129.*

Summary

In this chapter, we have outlined the anatomy and molecular controls of kidney and urinary tract development. These processes are prone to error, with genetic and environmental factors playing their parts. We suggest that, by studying the pathobiology of renal tract malformations, clues will emerge to suggest pathways toward novel therapies which will restore the balance toward more normal development.

Acknowledgments

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Conflict of interest statement

Neither author has conflicts of interest to declare.

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CHAPTER

2

Renal organogenesis in the lymph node microenvironment

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Introduction

The kidney is a multifunctional organ which performs several essential physiological functions, including excretion of metabolic wastes, osmoregulation, and hormone secretion. Due to their complex function and intricate structure, renal tissues are markedly vulnerable to injury. It is known that the mammalian kidney is capable of post-injury growth, albeit in a limited capacity. It cannot make new nephrons, and although some regeneration events take place following acute kidney injury (AKI), they are exclusively confined to the tubular epithelium [1]. However, tubular regeneration is frequently incomplete and accompanied by fibrosis and long-term loss of function. Severe and repeated episodes of AKI lead to chronic kidney disease (CKD). End-stage renal failure, also known as an end-stage renal disease (ESRD), is the final, permanent stage of CKD. Two million people worldwide suffer from ESRD, and the number of patients diagnosed with this disease continues to increase at a rate of 5%–7% per year [2]. ESRD patients require dialysis or a kidney transplant to survive. While life-saving in the short-term, dialysis is not a long-term substitute for a human kidney, performing only about 10% of kidney function, and it is associated with increased left ventricular mass, intramyocardial cell fibrosis, and capillary loss [3, 4]. Orthotopic kidney transplantation is the only definitive treatment option for ESRD patients [5]. It is however a major surgical procedure that has risks both during and after the surgery. For example, immunosuppressive therapy, which is administered to almost all kidney transplant recipients to prevent acute rejection and the loss of the renal allograft, is associated with an increased incidence of cancer and viral opportunistic infections [6]. Graft rejection and post-transplant complications resulting from over-immunosuppression are not the only limitations for kidney transplantation. Shortage of kidney donors is still a major limitation [7]. Indeed, despite efforts to promote the practice of organ donation, the resources available in many countries are currently insufficient to meet medical needs. As such, there is a growing need to find better, alternative therapies to treat varying forms of kidney disease. One such avenue involves the field of regenerative medicine to either replace, restore, or regrow renal tissues.

Recently, tissue organoids have emerged as sophisticated models for studying development and disease, and as potential sources for creating organ substitutes [8], including the kidney. However, kidney engineering is a challenging task due to the complexity of an organ which contains at least 26 different types of cells organized in a unique structure. A series of challenges must be faced. First, the identification and selection of the best-suited stem/progenitor cell type(s) for organoid generation. For years, the lack of definitive stem/progenitor cells in the adult kidney has been the major obstacle to renal repair and reconstruction [9]. With the development of the induced pluripotent stem cell (iPSC) technology, enabling the induction of pluripotency in mature somatic cells by treatment with defined factors [10], new opportunities to create patient-specific surrogate organs have emerged. Additional challenges of no less importance are the induction of substantial vascularization in kidney organoids in vitro, and the selection of a supportive transplantation site. This last objective is critical to allow the vascularized organoid to

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develop to functional maturity and perform important functions such as blood filtration, waste excretion, and essential substance reabsorption. We identified the lymph node—a secondary lymphoid organ which acts as a filter for foreign particles and cancer cells—as a surrogate niche for functional kidney organogenesis. The lymph node rapidly responds to accommodate increases in cellularity and has immediate vascular access, prerequisites for successful development of the kidney and its main function of blood filtration.

In this chapter, I will briefly discuss current strategies and challenges for engineering a functional kidney, and present our innovative strategy exploiting the lymph node. Our investigations seem to indicate that the lymph node might offer appropriate biophysical and biochemical cues to support kidney organogenesis.

Generation of tissue-engineered kidneys

Whole-organ tissue engineering has the potential to bridge the donor organ gap via the creation of artificial tissues by combining cells and matrices of natural or synthetic origin. Decellularization methodologies have been applied to produce whole organ-derived scaffolds by removing the cellular components while retaining all the necessary vascular and structural cues of the native organ [11]. However, the majority of described decellularization protocols show limitations in preserving the native structure of the extracellular matrix, mostly due to the harsh conditions required to remove cellular material [11]. At the moment, this technique only works with the heart in humans [12], and not the other major organs. Alternatively, supportive scaffolds can be produced from biomaterials including natural or synthetic polymers [13].

In the context of kidney, perhaps the most promising decellularization/recellularization strategy comes from the study of Song et al., which shows the generation of three-dimensional (3D) acellular renal scaffolds via perfusion decellularization of cadaveric rat, porcine, and human kidneys and their subsequent repopulation with endothelial and epithelial cells. Such kidney constructs were transplanted in an orthotopic position, and provided excretory function in vivo, following anastomosis to the recipient's renal artery and vein [14]. Although promising, translation of this technology beyond proof of principle will first require the isolation, differentiation, and expansion of specific stem/progenitor cell types from clinically feasible sources.

A better understanding of the molecular basis of normal kidney development in the human, coupled with the breakthrough in research on pluripotent stem cells, has recently made the generation and derivation of patient-specific kidney cells a future possibility [15]. It is now well accepted that the mammalian kidney develops from the intermediate mesoderm through reciprocal interactions of the ureteric bud (UB) and the metanephric mesenchyme [16]. Three multipotent progenitor cell populations can be distinguished: 1) the Hoxb7 + UB progenitors, giving rise to the collecting duct and the ureter; 2) the Six2 + nephron progenitors, giving rise to most of the nephron; and 3) the FoxD1 + stromal progenitors, giving rise to multiple nonepithelial cell types within the kidney, including vascular progenitors, mural cells, and resident fibroblasts [15]. These progenitors are likely exhausted once nephrogenesis ceases—before birth in humans [17], and at postnatal day 3 in mice [18]. Tremendous effort has therefore been made to generate self-renewing populations of both nephron and UB progenitors from pluripotent stem cells for therapeutic purposes. Artificially created renal progenitors can generate 3D multicellular mini-organ structures, or organoids, in vitro, differentiating into kidney components following culture in appropriate media supplemented with recombinant growth factors. Markers of mature nephron components (podocytes, proximal tubules, loops of Henle, and distal tubules) are detected in nephron progenitor organoids [19–23]. In some cases, organoids also contain a renal interstitium and an endothelial network [19]. Similarly, under appropriate 3D culture conditions, UB progenitors can be expanded in vitro as branching UB organoids. Aggregating 3D-cultured nephron progenitors with UB organoids have recently been attempted in vitro, resulting in a reiterative process of branching morphogenesis and nephron induction, similar to kidney development [24, 25]. Despite these remarkable advancements, there is a current lack of full maturation, well-developed vascularization as well as a limitation in scalability and reproducibility [26]. Transcriptomic studies have often revealed the immaturity of kidney organoids in terms of differentiation and functional capabilities [19]. Obtaining a mature organoid is crucial to minimize the chances of unwanted tissue differentiation after transplantation and to guarantee the developing tissue is functional. Kidney function not only requires the coordinated action of various cell types organized into specific segments, but also necessitates a special 3D structure allowing the countercurrent mechanism to concentrate urine [27]. While a scaffold might help achieve a better spatial orientation of tubules, the self-organizing processes that build organoids cannot be easily matched by engineering approaches relying on a scaffold. Controlling self-organizing systems is inherently difficult because they follow intrinsic developmental programs, and small changes in the initial conditions inevitably lead to variable outcomes. In light of these limitations, scaffold-free organoid-based bioprinting emerged as a promising alternative [28].

Traditional transplantation sites for experimental kidney tissue engineering

The selection of the best cell type(s) for kidney organoid formation and the generation of a specific spatial and histo-topographic arrangement of nephron tubules are not the only challenges to overcome in kidney tissue engineering. Even assuming the most recent cell technologies would give rise to functional and well-aligned nephrons, where should they be transplanted? The possibility of implanting developing nephrons beneath the renal capsule of a postnatal or an adult host kidney has been studied since the early 90s [29]. However, this approach is not feasible in the clinic for at least two reasons. First, significant differences exist between murine and human kidney anatomy inasmuch as the human kidney capsule and parenchyma cannot be as easily separated as in rodents to permit cell transplantation [30]. Second, the fibrotic and inflammatory milieu of a chronically injured human kidney might not be an ideal niche for functional kidney organogenesis.

Organogenesis in an ectopic site was proposed as an alternative method to provide auxiliary organ function [31, 32]. Several ectopic sites were investigated in animals as transplantation sites for renal primordia (metanephroi), including the anterior eye chamber [33], the omentum [34], the retroperitoneal fat [35], the abdominal aorta [36], the paraaortic area [37], and the epididymis [38]. Metanephroi transplanted under the renal capsule [39] or in such ectopic sites [33–38] enlarged, become vascularized, formed glomeruli and tubules able to concentrate urine to a degree. However, the developing kidney would grow and survive for only a short time, unless an end-to-end anastomosis to the host ureters was performed [35, 36, 38]. Ureteroureterostomy between the transplant and native ureter prolonged short-term survival of anephric rats [35], and raised blood pressure in acutely hypotensive rats [38]. Although promising, these results point to several clinical limitations. Except for the omentum, none of the analyzed sites has the potential to reach the clinical practice. The greater omentum has already created significant interest for its potential as a site for pancreatic islet transplantation. A clinical trial investigating outcomes in patients who receive islet transplants to this site been launched [40]. However, major complications related to the use of the omentum include postoperative adhesions and intestinal obstruction [41, 42].

The lymph node microenvironment as an alternative site for kidney tissue engineering

The lymph node is a secondary lymphoid organ that has a unique ability to act as a bioreactor, allowing for substantial expansion of white blood cells during various conditions. This property is exploited by cancer cells, which often transit via the lymph node where they efficiently grow before disseminating to distant organs. Our group has used this growth-permissive environment to grow several tissues, including the kidney [43–51]. Following early kidney rudiment transplantation, robust kidney tissue forms in which nephron-like structures can be identified by molecular markers. We observed that host vasculature connects to the engrafted tissue allowing host blood flow through glomeruli, strongly suggesting the physiological function of the developing nephrons. Notable features of this engraftment technology make it a unique tool in which to study the organogenetic potential of pluripotent stem cell-derived kidney tissues and might have future clinical implications. In the paragraphs below, I will discuss our results of kidney organogenesis in this unique site.

Engraftment of mouse and human fetal primary tissues in the lymph node

By using fragments of green fluorescent protein (GFP) transgenic mouse metanephric (E14-15.5) kidneys, we first showed successful nephron maturation inside a host mouse lymph node [47, 48]. Renal tissues were harvested from C57BL/6 GFP + transgenic embryos, isolated from UBs, minced, and injected directly into a single jejunal lymph node of adult wild-type C57BL/6 mice [47]. Following 3 weeks, recipient mice were sacrificed, lymph nodes were collected, and histologically examined [47]. Although some s-shaped bodies (glomerular and nephron precursors) could still be identified, the grafts contained several mature glomeruli, expressing CD31 + endothelial cells, and podoplanin + podocytes [47]. Engrafting kidneys also showed cytokeratin-8 (K8) + rudimentary tubules as well as tubular erythropoietin expression [47].

By 6 weeks, mature nephrons consisting of mature glomeruli as well as renal tubules were observed [47]. Moreover, erythrocyte presence inside the mature capillary tuft indicated probable glomerular filtration ability [47]. To assess tubular maturation, we performed RT-PCR analysis for the presence of different urea transporters [47]. UT-A transporter immunoreactivity appears first in E16 kidneys and strongly increases after birth [52]. Interestingly, we found all UT-A family members analyzed to be expressed at the mRNA level in 6-week grafts, suggesting tubular epithelial maturation and likely de novo acquisition of urea-reabsorption [47].

Time-course experiments indicated that healthy nephrons could be found until the 12th week post-transplantation [47]. Over time, the production of fluid waste could result in degeneration of the ectopic graft [47]. Indeed, urine-like fluid-containing cysts and glomerular alterations were observed in grafts at later transplantation stages [47].

Importantly, we found that both bone marrow hematopoietic and stromal cells supported ectopic kidney organogenesis [47]. Not only did the lymph node furnish the developing tissue with host cells, but the lymph node also provided growth and homeostatic signals, since a decrease in native renal mass could push maturation of the ectopic graft [47].

Later, we demonstrated that the lymph node lends itself well as a niche to also grow human primary kidney rudiments [49]. We used human fetal kidneys between 14 and 23 weeks of gestation for transplantation [49]. While there are well-developed glomeruli and tubules at these stages, there is also a nephrogenic zone just underneath the renal capsule, containing different progenitor populations [53]. We used this zone to obtain tissue fragments to be transplanted into the lymph node. Athymic Balb/C mice were chosen as immunodeficient transplant recipients to avoid T cell-mediated rejection [54]. Three weeks after transplantation, an initial lymph node examination suggested tissue engraftment and no evidence of rejection [49]. Staining of the engrafted lymph nodes for the mouse pan-leukocyte marker CD45 suggested maturing human kidney tissues were devoid of infiltrating immune cells [49]. Further analyses indicated that similar to mouse embryonic kidney, human fetal kidneys acquired markers of mature renal structures, had functional glomeruli, tubules, and possessed the hormonal functions of erythropoietin and cytochrome P450 family 27 subfamily B member 1 (CYP27-B1, also known as 1alpha-OHase, necessary for the conversion of calcifediol to active calcitriol) [49].

Partial 3D reconstruction from the digital images of hematoxylin and eosin-stained serial sections of an 8-week graft confirmed the presence of glomerular and tubular structures still properly arranged [49]. Moreover, 10,000 MW Dextran uptake in proximal tubules of lymph node grafts at even further stages (11 weeks) indicated long-term human kidney tubular functions [49].

Interestingly, the lymph node-engrafted nephrons could rely on a remarkable vascular network originating from both host- and graft-derived vascular cells [49]. Host endothelial cell infiltration occurred as early as 1 week after transplantation, when human donor- and mouse host-derived endothelial cells coexisted [49]. By the 3rd week, glomeruli were entirely re-vascularized by mouse endothelial cells, and the caliber of mouse extraglomerular blood vessels reached its maximum level by 8 weeks [49].

Collectively, these data suggested the feasibility of generating human nephrons with mature glomeruli and physiologically and anatomically distinct tubular segments inside a mouse lymph node.

Engraftment of isolated or artificially created nephron progenitors in the lymph node

Since the lymph node provided a supportive niche for the engraftment, vascularization, and function of fragments from both mouse [47, 48] and human fetal primary kidney tissues [49], we next asked whether it could also support functional maturation of isolated or artificially created nephron progenitors.

Single-cell suspensions of embryonic kidney failed to properly differentiate in the lymph node, even though they were comprised of SIX2 + cells [47]. When we co-transplanted SIX2 + cells and mouse embryonic kidney fragments (to act as a scaffold and inducer for SIX2 + cells) into the lymph node, few progenitor cells incorporated into the engrafted renal tissues and they were unable to mature into meaningful structures [49]. We hypothesized that nephron progenitors must be grown as organoids and partially differentiated before transplantation for efficient kidney organogenesis in vivo.

We therefore, grew SIX2 + mouse nephron progenitors in 96-well U-bottom low-attachment well plates in NPSR culture medium [23]. The resulting spherical aggregates displayed a robust and homogeneous expression of nephron progenitor markers [49]. Aggregates were transplanted in the lymph node following a short 3-day pulse of 4.5 μM CHIR99021, an extremely potent glycogen synthase kinase 3 inhibitor and activator of the Wnt/β-catenin signaling pathway. One week after transplantation, we observed e-cadherin (CDH1)−/lotus tetragonolobus lectin (LTL)+and CDH1 +/dolichos biflorus agglutinin (DBA)+tubular structures, indicating in vivo maturation of mouse nephron progenitors into proximal and distal tubules, respectively [49]. Moreover, the grafts contained several host CD31 + endothelial cells, often in contact with Wilms' tumor 1 (WT-1)+aggregates (potential primitive glomeruli) [49]; this showed the feasibility of obtaining vascularized glomerular-like structures from mouse nephron progenitor-derived kidney organoids in the lymph node.

In parallel, we evaluated the behavior of human iPSC-derived kidney organoids inside the lymph node. We used the protocol developed by the Little laboratory for human iPSC differentiation [19]. This protocol allows the generation of organoids containing derivatives of all major renal progenitors, including patterned and segmented nephrons,

collecting ducts, renal interstitium, and endothelium. However, nephrons within these organoids are not fully differentiated and lack functioning vasculature. Transplanted organoids in the lymph node engrafted and showed signs of host endothelial cell infiltration in 1 week [49]. A few mouse CD31 + cells localized within WT-1 + aggregates [49]. Several LTL + tubules were also observed [49]. While LTL was present prior to transplantation, there was no CD31 or WT-1 expression in vitro. Importantly, 6 weeks after transplantation we could detect glomerular-like structures containing mouse endothelial cells and mature podocytes adjacent to presumptive proximal tubules [49].

Taken together, our data showed that the lymph node has a unique ability to act as a bioreactor, allowing for differentiation of pluripotent stem cell-derived kidney tissues, as suggested by early engraftment and vascularization of nephron progenitors and human iPSCs that were directed toward a renal fate in vitro (Fig. 2.1).

Lymphatic cues supporting kidney organogenesis

Similar to the lymph node, the omentum supported the maturation of GFP-expressing metanephric kidneys [50]. Our investigations indicated the host lymphotoxin-beta receptor (LTβR) pathway generates angiogenic cues to support kidney organogenesis in both sites [50]. Our hypothesis that LTβR could impact kidney organogenesis in both omentum and lymph node sprang from LTβR's central role in the development [55, 56] and homeostasis [57] of lymphoid organs. LTβR is widely expressed on the three major CD45- lymph node stromal cell populations—fibroblastic reticular cells (FRCs), lymphatic endothelial cells (LECs), and blood endothelial cells (BECs)— and is important for several adult lymph node functions [57]. LTβR signals on FRCs regulate lymph node vascular endothelial growth factor levels and, in turn, lymph node endothelial cell proliferation [58]. Likewise, LTβR triggering on lymph node endothelial cells impacts vasculature development [59]. We found lymph node and omentum share similar FRC/LEC/BEC flow cytometric profiles [50]. Moreover, we identified LTβR +/PDPLN + and LTβR +/PDPLN- cell subsets in omental cell suspensions [50], indicating the existence of FRCs and BECs mediating lymphotoxin signals in this site as well. Interestingly, glomeruli maturing in both lymph nodes and omenta contained, during the early stages of engraftment, endothelial cells expressing the nuclear factor κB-inducing kinase (NIK) [50], a downstream target, among others, of LTβR [60] (Fig. 2.2A). Given an established role for NIK in neovascularization [61], and the presence of host endothelial cells in ectopic kidney grafts, we hypothesized that host LTβR/NIK signaling facilitated glomerular vascularization inside the lymph node. In accordance with our hypothesis, engrafted glomeruli were found to be embedded in a host LTβR reactive cellular scaffold, compatible with ER-TR7 + FRCs (Fig. 2.2B).

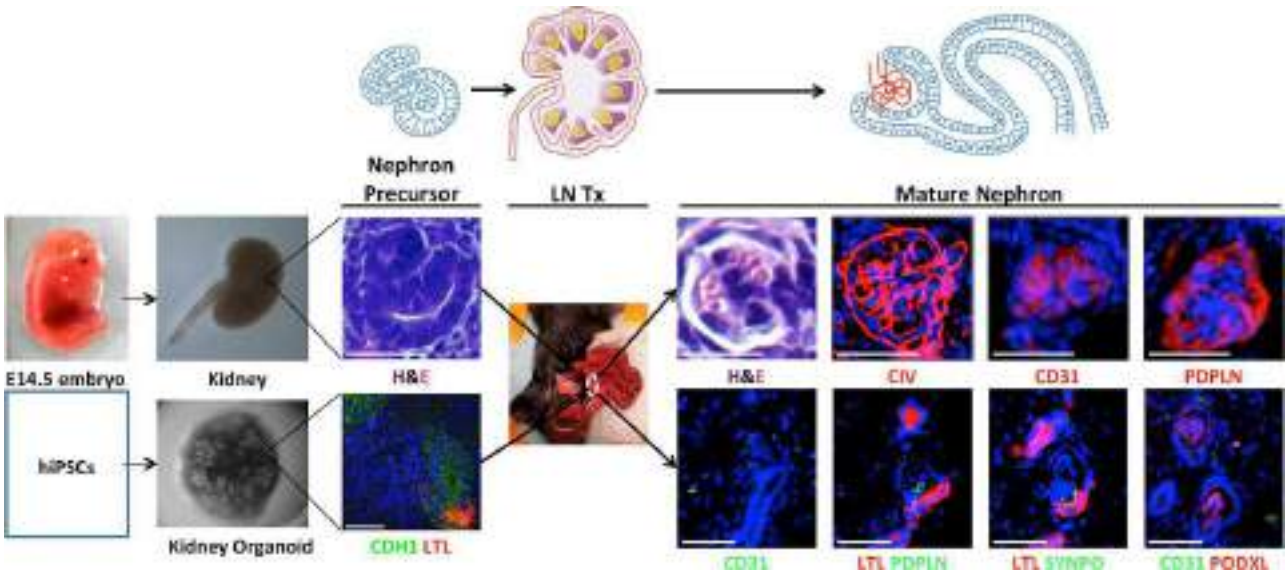

FIG. 2.1 Kidney engraftment in the lymph node. *From top to bottom:* Schematic view of transplantation of a nephron precursor into a lymph node and its maturation into a vascularized nephron; isolation of mouse metanephric kidney and transplantation (Tx) into the lymph node (LN, *left*), and generation of vascularized glomeruli 3 weeks later (*right*); establishment of human iPSCs-derived organotypic kidney cultures in vitro (*left*) and their differentiation in nephron-like structures inside the lymph node (*right*). Maturing glomeruli were stained with hematoxylin and eosin (H&E), a constituent of mesangial matrix (CIV), a blood vessel marker (CD31), or podocyte-specific markers (PDPLN, SYMPO, PODXL). LTL was used to stain proximal tubules. Nuclei were counterstained using Hoechst (*blue*). Scale bars = 50 μm.

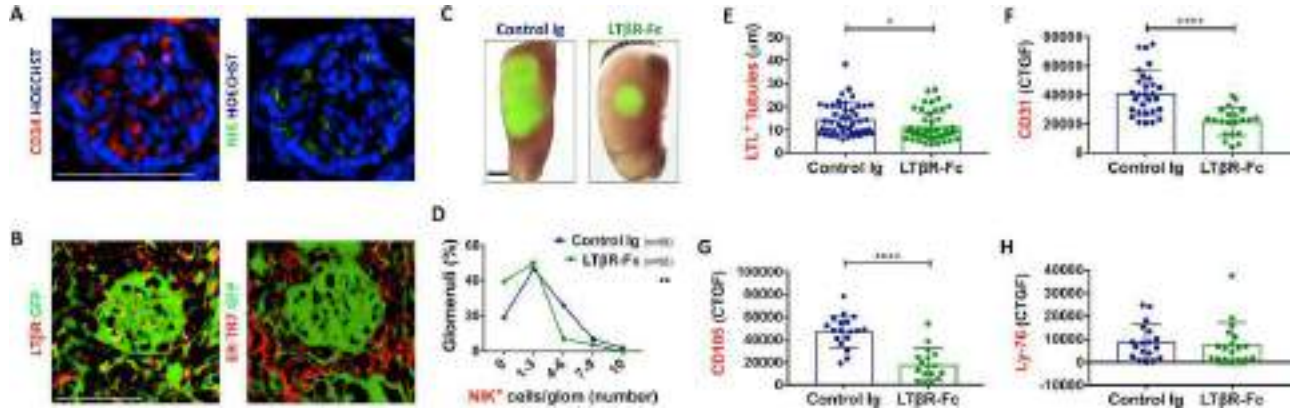

FIG. 2.2 Vascularization of glomeruli engrafted in the mouse lymph node is driven by host LTβR/NIK axis. (A) Representative immunofluorescence stain for CD34 (endothelial cell marker, *red*) and NIK (*green*) in an engrafted wild-type (GFP-) glomerulus 3 weeks after transplantation. (B) Representative immunofluorescence stain for LTβR (*red*) and ER-TR7 (lymph node fibroblastic reticular cells, *red*) in engrafted GFP + glomeruli 3 weeks after transplantation. (C) Merged fluorescence/bright-field images of whole mount kidney-bearing lymph nodes isolated from either control immunoglobulin (Control Ig)- or LTβR-Fc-treated mice (GFP + areas indicate kidney engraftment). (D) Line graph showing the percentage of NIK-expressing cells in engrafted glomeruli. Glomeruli were divided into five groups based on the number of NIK-expressing cells (0; 1 to 3; 4 to 6; 7 to 9; or 10 positive cells/glomerulus). (E) Scatter dot plot with bar graphs ± SD showing the length of LTL + tubules in kidney grafts as in C, measured across sections, using ImageJ. Each dot represents a tubule. (F–H) Scatter dot plot with bar graphs ± SD presenting the corrected fluorescence intensities of glomerular (CTGF) CD31, CD105 (neoangiogenetic marker), and Ly-76 (erythroid cell marker) in kidney grafts as in C, measured using ImageJ. Each dot represents a glomerulus. Scale bars = 25 μm (A and B); 500 μm (C). $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ (unpaired Student's *t*-test, D-G).

By transplanting kidney rudiments either in the lymph nodes of mice undergoing LTβR antagonist treatment (LTβR-Fc) or in the omenta of *Ltbr*$^{-/-}$ mice, we confirmed that host LTβR signals are crucial for obtaining a well-vascularized kidney graft [50]. Indeed, defective host LTβR signaling correlated with decreased expression of endothelial and angiogenic markers in the transplanted tissues as well as structural alterations [50]. Specifically, LTβR-Fc treatment resulted in the reduced expansion of transplanted GFP + metanephric kidneys (Fig. 2.2C) and decreased numbers of NIK + glomerular cells (Fig. 2.2D). LTβR-Fc treatment also resulted in decreased average LTL + tubule length, calculated in cross-sections (Fig. 2.2E). The extent of vascularization/angiogenesis was evaluated by staining for CD31 and the neoangiogenic marker CD105. The intraglomerular CD31 and CD105 fluorescence intensities were quantified and a significantly weaker intensity was found for both markers in glomeruli of LTβR-Fc-treated mice (Fig. 2.2F and G).

Although the number of glomerular endothelial cells expressing the LTβR target NIK decreased in the absence of a functional LTβR, the results from the transplantation of kidney rudiments in the omenta of *Nik*$^{-/-}$ mice indicated that host NIK is not required for successful ectopic kidney organogenesis [50]. This result might suggest either the existence of different targets downstream of LTβR leading to angiogenesis or the activation of compensatory angiogenic pathways in the absence of NIK.

NIK and subsequent non-canonical NF-κB signaling regulate both inflammation-induced and tumor-associated angiogenesis [61]. From this perspective, kidney angiogenesis in lymphoid microenvironments shares features with pathological angiogenesis. However, the progressive reduction in NIK-expressing cells that was observed in glomeruli engrafted in lymph nodes and omenta [50] seems to suggest a tight regulation of angiogenesis in the ectopic kidney grafts, where NIK likely elicits a transient signal.

Conclusions

Chronic and end-stage kidney disease impose a large burden on human health worldwide. Projected prevalence trend lines are not comforting, suggesting an urgent need to explore new approaches to overcome limitations associated with traditional interventions.

Kidney engineering is a challenging task. The challenge is multi-fold: (1) understanding the fundamental principles underlying cellular organization in the developing human kidney; (2) figuring out ways to re-create such cellular organization in vitro; (3) designing scaffolds that provide cells with specific cues or identifying supportive in vivo niches; (4) defining strategies for achieving functional vascularization; (5) assessing and evaluating organ function; (6) scaling up to develop functional human-sized preclinical models.

Significant effort has been made to develop vascularized organoid cultures that could mimic the renal primordia [62, 63]. Indeed, there is evidence to suggest that transplantation of renal primordia has advantages with respect to transplantation of developed kidneys, including a quicker vascular integration via anastomoses with the host's vasculature, and a reduced risk of rejection following transplantation across immunologic barriers [64]. Our published results reviewed in this chapter indicate that thanks to its high vascularity, the lymph node microenvironment might offer a surrogate niche for the successful maturation of renal primordia [43–51]. Indeed, in this site, primary mouse and human embryonic kidney fragments, as well as organoid cultures derived from both mouse nephron progenitors and human-induced pluripotent stem cells, differentiated into mature renal structures with excretory, homeostatic, and endocrine functions.

While the degree of kidney graft function observed in our studies supports further investigation into lymph node research for kidney reconstruction, we are still a long road away from engineering a functional kidney suitable for clinical practice. The human body relies on a pair of kidneys, each containing about one million nephrons. With improvements in scale and structure, our lymph node-based strategy may provide a functional replacement for failing kidneys as an alternative to dialysis for the treatment of kidney failure. This goal will first require building a kidney organoid in which a satisfactory number of nephrons are correctly aligned and connected so that a single path for urine is obtained. Urine could later be drained from the ectopic kidney graft through surgical and/or engineering techniques. While this goal seems unrealistic, the lymph node could be better exploited to provide an auxiliary aid during renal failure. For example, the lymph node could be manipulated to establish a self-sustaining tissue that secretes erythropoietin. Erythropoiesis-stimulating agents including epoetin alfa and darbepoetin alfa are widely used to slow the progression of renal failure [65]; however, such treatments are costly [66]. Alternatively, stromal cells from the lymph node might be exploited to create an artificial scaffold for renal progenitor cells.

In conclusion, the lymph node might offer unprecedented opportunities to study stem cell maturation and function, as well as provide a unique organogenesis site for therapeutic purposes. Improved organoid engineering and a better understanding of the unique properties of the lymph node and its cells might have implications for the treatment of several forms of kidney disease.

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CHAPTER

3

In vivo clonal analysis and the kidney: Implications to regenerative nephrology

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Introduction

During the past decades, our understanding of the way the kidney develops, functions, and responds to damage has grown exponentially. This has resulted from the combined use of various models, both in vitro and in vivo. Paramount to this process was the use of genetically manipulated mouse models (i.e., transgenic mice), representing the most studied organism and the basis for a significant portion of the knowledge gained about the mammalian kidney. In particular, these models are an effective means of delineating cellular lineages within the kidney during both steady state and disease and understanding how this may be translated to regenerative nephrology. Herein, we review the basic concepts of in vivo lineage tracing and present how this tool has provided answers to some of the most fundamental and important questions in kidney growth and regeneration in fetal and adult life.

Principles of in vivo lineage tracing

In its basic form, a lineage-tracing experiment involves the labeling a single cell, which results in the labeling of all its daughter cells. This way, one can analyze the entire cellular hierarchy that stems from the originally labeled pool of cells. Nowadays, this is carried out by expressing a recombinase gene only in the cells of interest, which in turn leads to the expression of a reporter gene (e.g., fluorescent protein), thereby irreversibly marking the cell [1]. Once the cell divides, the reporter gene is transmitted to its progeny, which are now also labeled, and so on. The cell-type specificity is achieved by placing the recombinase gene under the control of a promoter of a gene that is specific to that cell (e.g., podocin to mark podocytes) [2]. More sophisticated versions of this technology allow researchers to control not only the location of marker expression but also the timing, such that cellular lineages can be traced only from a specific point in time (e.g., only postnatally). This results in significant flexibility and an ability to explore various biological questions [2]. In fact, it is currently possible to trace the clonal evolution of single cells in vivo, thereby evaluating fundamental concepts in stem cell biology. Nonetheless, the experimental design is crucial in obtaining reliable answers to these questions. In particular, appropriate selection of the promoter and reporter is highly important [2].

The most widely used system for in vivo lineage system is the Cre/LoxP. In this system, the bacterial enzyme Cre-recombinase is expressed only in the cells of interest (i.e., through the use of a promoter of choice) and leads to the labeling of these cells by activating a reporter gene by its interaction with the LoxP sites [3]. The latter are short (34 bp long) sequences artificially placed on either side of a "stop" sequence inserted into the reporter construct, thereby preventing its expression. Cre excises the DNA fragment that lies between the LoxP sites, which leads to the labeling of the cells. This way, only cells that express Cre (i.e., where the promoter is active) are labeled. To gain control not just over which cells to label, but also when to label them, modified promoters can be used, in which the Cre enzyme

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is amalgamated to a modified version of the estrogen receptor (ER) that is devoid of the ability to bind estrogen and binds to tamoxifen. Later advancements improved the sensitivity of the mutated ER receptor toward tamoxifen even further. Using this CreER^{T2} chimeric protein, the labeling of the desired cell type takes place only once exogenous tamoxifen is administered by the researcher. Notably, the development of a very large selection of Cre driver mouse lines now enables the analysis of a wide array of cell types in the kidney [3]. In addition, the system is highly flexible and can be used in several ways so as to address specific questions. These include: (1) temporal control over the time point of initial cell marking, which allows following the cellular fate of the same population at different developmental stages; (2) usage of a promoter of a stem cells gene (e.g., Six2) to either trace its lineage or explore different aspects in the differentiated progeny that result from it (e.g., tubular epithelia); and (3) modification of tamoxifen doses to mark single cells or cell populations [4].

One of the crucial steps in successful lineage tracing is the proper selection of a promoter of interest. Broadly speaking, these can be divided into endogenous promoters, present in the native mouse genome, and exogenous, artificially inserted promoters. Each promoter has its own advantages and disadvantages, and careful interpretation of the obtained observations, as well as the use of more than one promoter, is usually warranted [2].

Lineage tracing for elucidation of critical steps in nephrogenesis

One of the most important contributions of lineage-tracing technologies to the field of kidney stem cells has been the unraveling of differentiation processes during embryonic kidney development [5, 6]. This not only resulted in better understanding of the way mammalian kidneys develop but has also provided the essential information required to recapitulate these fundamental processes in vitro, for instance by inducing human pluripotent stem cells into kidney organoids reminiscent of fetal kidney.

For example, several lineage-tracing studies were used to characterize the relationship between the transcription factor OSR1 and nephrogenesis. Initially, through the use of a temporally regulated Cre-LoxP system, OSR1$^+$ cells were traced during mouse development, using different starting points as dictated by the administration of tamoxifen [7]. Remarkably, while at early time points (e.g., E7.5), OSR1$^+$ cells were found to represent the ancestral cells of most cell types in the kidney (i.e., nephrons, ureteric epithelia vasculature, and interstitium), their differentiation potential became progressively narrower, with OSR1$^+$ cells at E11.5 giving rise only to nephrons [7]. A later study expanded these findings and revealed that at E8.5, OSR1 + cells are already separated into at least two, differently committed populations of stem cells [8]. The first, located in the anterior intermediate mesoderm, is destined to give rise to the ureteric bud. The second, located in the posterior intermediate mesoderm, gives rise to the metanephric mesenchyme, which is the primordial tissue of the nephrons.

One of the most widely used promoters in the study of kidney stem cells is the Six2 promoter. Six2 represents perhaps the most specific marker of the cap mesenchyme (CM), which is the progenitor population of all tubular epithelial cells, from podocytes through distal tubules (excluding the collecting ducts, which are derived from the ureteric bud) [9, 10]. Hence, lineage tracing of Six2-expressing cells has resulted in some of the most important insights in the field of kidney stem cells. Through the use of low-dose tamoxifen given to Six2-CreER^{T2} mice (i.e., mice expressing the Cre recombinase under the control of the Six2 promoter), during fetal development, labeling of single CM cells was achieved. By analyzing the kidney after the completion of nephrogenesis, it was possible to decipher exactly which cell types are derived from the Six2 population, and whether or not single Six2$^+$ cells can give rise to multiple cell types (i.e., are multipotent). Indeed, it was demonstrated that the entire nephron, from podocytes to distal tubule, was labeled, providing compelling evidence to the multipotent nature of the Six2-expressing population [10]. Promoters of other genes demarcating kidney progenitors, such as Cited1 (CM) [11, 12] or Ret (ureteric bud) [11, 12], can similarly be used to trace specific populations within the developing kidney.

Postnatal lineage tracing for elucidation of homeostatic and damage-response mechanisms in the kidney

Relying on the same type of transgenic mice, Humphreys et al. analyzed the postnatal kidney to conclusively show that the regeneration of damaged tubular epithelial cells following acute kidney injury relies completely on cells from within the tubular epithelium (as opposed to extra-renal or nonepithelial cells) [13]. This has settled an important debate in the field, surrounding the question of whether or not extra-renal cells (e.g., bone-marrow-derived cells) contribute to the repair of injured kidneys. Equally important, the same study proved that the postnatal kidney

is devoid of a Six2-expressing pool of multipotent kidney stem cells, both during steady state and after injury. This strongly suggested that the adult kidney relies on unipotent precursors for the generation of new tubular cells, a finding that was later shown using a different type of transgenic mice (see in the following section). Despite the importance of this seminal work, several caveats must be taken into consideration. These include the inability of this system to exclude the presence of a non-Six2$^+$ progenitor population within the kidney, potential interspecies differences, and the use of a single model of damage.

By focusing on a different population of progenitors, the FoxD1$^+$ stromal progenitors, it has been possible to determine the cellular origin of myofibroblasts in kidney fibrosis, which is the final common pathway in a myriad of chronic kidney diseases [14]. Two potential origins were initially suggested for this mesenchymal population, namely tubular epithelia (i.e., via the process of epithelial to mesenchymal transition [EMT]) or resident mesenchymal kidney cells. Lineage tracing of the descendants of the fetal FoxD1$^+$ population showed that epithelial cells do not participate in the formation of kidney scars and fibrosis. Rather, mesenchymal cells derived from FoxD1$^+$ progenitors are the source of this phenomenon, a finding with significant ramifications for the treatment of chronic kidney disease.

Lineage tracing in the adult kidney

Lineage-tracing experiments employing promoters of terminally differentiated cell types in the kidney represent another valuable approach, capable of uncovering cellular dynamics in the adult kidney. By analyzing the behavior of cells expressing the injury marker Kim1, it was demonstrated that kidney repair after ischemic injury relies heavily on de-differentiated epithelia, which undergo limited clonal expansion to replace necrotic cells, and involves induction of the transcription factor Foxm1 [15]. Another study sought to answer another important question in the field of renal regeneration: does the repair of injured kidneys involve resident stem cells? To answer this question, a transgenic mouse strain, in which cells expressing the specific proximal tubule marker SLC34a1 are marked, was exposed to kidney injury [16]. The assumption was that if only fully differentiated cells contribute to renal repair, then only labeled cells would be evident following injury. In contrast, if there is a contribution from an intrinsic renal pool of stem cells/progenitors, then dilution of the marked population will be noted. Pointing against the presence of such a population, no marker dilution was evident. While this study beautifully demonstrates how lineage-tracing studies can be used to elegantly resolve cardinal issues in kidney biology, it should be noted that the conclusion relies on the premise that a subpopulation of progenitors that also express the SLC34a1 marker does not exist, which is difficult to prove.

Multicolored transgenic mice

Another type of lineage tracing allows the random expression of different ratios of several fluorescent proteins, each with a different color, in individual cells, resulting in stochastic distribution of cells with different colors. Such multicolor, originally limited to neuronal lineages (the so-called "brainbow" mouse), now allows using this system to study various aspects in different tissues and cell types, although multicolor reporters are optimal when used in combination with inducible Cre models [2].

A multicolor lineage-tracing system was used to determine whether specific nephron segments (e.g., proximal tubule, loop of Henle, and distal tubule) are regenerated after damage or during steady state by cells from within the same segments [17]. Using the rainbow mouse, each tubular cell was randomly marked with one of the four colors (green, blue, red, or yellow) upon tamoxifen injection. Using low-dose tamoxifen, resulting in less than 1% frequency of recombination, single cells were labeled, thereby allowing analysis of clonal evolution at the single cell level. Following 7 months, the kidneys were removed and analyzed for the relative abundance and spatial distribution of the differently labeled cells. This demonstrated the presence of single cell-derived (unicolored) slowly expanding clones, which were restricted within their specific segments. This study clearly demonstrated that the mechanism behind kidney tubular maintenance and response to injury involves unipotent precursors. In a broader sense, clonal nephron cells are responsible for cell replacement in the kidney by cell division and as we previously termed this, harbor progenitor characteristics. Analysis of nephron clones that arise from Axin2$^+$ cells (cells in which a Wnt signal is transduced) showed a larger progeny, and hence, clonal Axin2$^+$ cells may represent replication-competent nephron cells that are activated by a neighboring Wnt signal as shown, for instance, in the liver [18]. Whether the latter represent adult epithelial progenitors or

differentiated cells stochastically taking on a regenerative phenotype is yet to be determined. Nonetheless, these findings have important implications for renal regenerative medicine. Namely, they show that in contrast to the fetal kidney, which relies on a multipotent pool of stem cells for the generation of nephrons [5,19,20], the adult kidney employs a completely different mechanism, whereby segment-specific precursors act to replenish naturally shed or injured epithelia. From the translational perspective, this might mean that rather than attempting to establish multipotent kidney stem cells, derivation of several, segment-specific precursors, possibly administered together, might be the preferred route.

Lineage tracing in the service of renal regenerative medicine

We previously demonstrated that NCAM1$^+$ human fetal kidney cells cultured in serum-free medium represent a mesenchymal cell population expressing nephron progenitor genes (e.g., *OSR1*, *SIX2*, and *CITED1*) and harbor multipotent differentiation potential toward renal epithelia [21–23]. Accordingly, the NCAM1$^+$ cells improved kidney function in mice with CKD [24]. Nonetheless, the NCAM1 fraction also contained nonepithelial lineages, and so to pinpoint the desired population, we identified the different fractions characterized by the combinatorial expression or nonexpression of NCAM1, CD133, and EpCAM. Specifically, we were able to show that the NCAM1$^+$CD133$^-$EpCAM$^-$, NCAM1$^+$CD133$^-$EpCAM^{dim}, NCAM1$^+$CD133$^-$EpCAM^{bright}, and NCAM1$^+$CD133$^+$ populations correspond to the CM, early differentiating CM, renal vesicle and later differentiating steps (c/s-shaped bodies and tubules) [25]. Hence, multipotent human fetal kidney-derived nephron progenitors can be established using a simple marker-based system.

In contrast, evidence from in vitro cultures of adult kidneys directly reflects the mechanisms of cell genesis in the adult kidney, as discussed earlier, namely expansion of segment-restricted cells. For instance, when we analyzed single cell-derived clones derived from the adult human kidney and grown ex vivo, each clone was seen to belong to a specific segment, underscoring the clear lineage boundaries within the kidney [26]. Interestingly, a comparison between early clones to bulk cultures established from the human adult kidney showed dissimilarities: early clones overall reduce adult renal identity markers but at the same time can be observed to harbor identities of multiple kidney segments as well as overexpress renal developmental markers. However, a comparison among growing clonal cultures showed clear nephron lineage distinction [26]. The same phenomenon was noted upon generation of multicolored murine kidney spheroids in which individual colors were always restricted to a specific nephron linage [17] as well as kidney spheroids from normal human epithelia [27,28]. These again demonstrated lineage-restricted potential in both culture and upon in vivo tubulogenesis. Notably, the same principles apply to nonepithelial lineages in the kidney, such as endothelium and perivascular cells. For instance, while whole human fetal kidney grafts are capable of generating donor-derived vessels upon transplantation into a murine host [19], transplanted adult kidney cells rely on the host vasculature for proper graft perfusion. Relying on these fundamental insights, we recently demonstrated that through combined injection into immunodeficient mice of renal tubule-forming cells and vessel-forming cells (endothelial colony-forming cells and mesenchymal stem cells), renovascular units are generated, harboring both differentiated renal tubules and donor-derived vessels, capable of connecting to the host circulation [29]. This again exemplifies that when using postnatal cell types, the separate formation of each lineage from its own precursors is needed to obtain a functional graft composed of all the desired cell types.

Consideration and limitations

In vivo lineage-tracing systems involve several important considerations which must be taken into account to obtain reliable results. First, a potential caveat in these systems is that recombination might take place even without induction. Such "leakiness" may distort the observed lineage relationships [2]. Hence, prior to carrying out the actual experiment, calibration studies assessing the extent of leakiness across different tissues have to be performed. Notably, the efficiency of labeling can also be influenced by a myriad of factors, including route of drug administration and mouse weight and body composition, and all of these factors have to be accounted for [30]. Second, as presented in some of the studies earlier, the efficiency of Cre activation and consequently the frequency of labeled cells are dependent on the dose of the administered drug (e.g., tamoxifen). This, in turn, can affect the observed results, and thus has to be selected according to the biological question that is addressed. For instance, if one is interested in studying clonal evolution at the single cell level, low dose is required. The same strategy, however, will not be suitable when trying to study the cellular dynamics of an entire population expressing a specific marker.

Summary

In vivo lineage tracing is a unique and important tool in the toolbox of stem cell researchers. The use of this strategy allowed the uncovering of some of the most important notions in the field, surrounding both kidney development and the mechanisms of renal response to injury. Despite being rather complicated and costly, through a combination of a clear biological question and carefully designed study, this methodology is irreplaceable in its ability to study cellular dynamics in vivo and providing important answers to the way normal and diseased tissues behave.

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CHAPTER

4

Nephrogenesis in malnutrition

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Introduction

In 1986, the epidemiologist David Barker first proposed the theory of fetal origins of adult disease. At that time, Barker published his findings that proposed a direct connection between prenatal malnutrition and coronary heart disease [1]. This theory by Barker gave rise to the fetal origins hypothesis, which proposed adverse events (such as maternal malnutrition) that occur during gestation can have significant effects on the development of the fetus and its health throughout life. This means the environment that the fetus develops in while in the mother's womb plays a decisive role in determining the risk for disease in the fetus after it is born and during its maturation through adulthood, a concept that is referred to as fetal programming. Maternal malnutrition during pregnancy affects various aspects of fetal organogenesis (including kidney development) through epigenetic modifications, altered gene expression and cell signaling, reduced availability of cellular building blocks, increased oxidative stress, and reduction of the critical stem cells needed for organ development.

Various animal models have demonstrated that maternal malnourishment (including maternal protein and/or caloric restriction) during gestation results in offspring that experience impaired kidney development, a significantly reduced nephron count, renal dysfunction, and high blood pressure [2]. However, much of these impairments can be prevented with vitamin, mineral, or amino acid supplementation during gestation, thus further exhibiting the importance of optimal nutrition in kidney development [3–5]. While human observational cohort studies have also similarly demonstrated maternal malnourishment impacts offspring health, one famous example is that of the Dutch from World War II. From 1944 through 1945, much of the Netherlands were under famine conditions because of the German blockade that prevented food supplies from reaching many of the Dutch citizens. Dutch women who were pregnant during this famine gave birth to babies that showed over the course of their lives a significantly enhanced risk for the development of variety of diseases, including cardiovascular disease and renal dysfunction (reviewed by Painter et al.) [6].

This chapter discusses the specific effects that maternal malnourishment has on kidney development in the fetus and the subsequent effects on renal function after the birth of the fetus. This chapter also discusses what is currently known about the role and effects that specific macronutrients (proteins, lipid, fats, and fatty acids) and micronutrients (vitamins and minerals), and their deficiency, have on kidney development and function.

Kidney development and nephrogenesis overview

At week 5 of gestation in humans (embryonic day 10.5 in mice), nephron formation begins, a process referred to as nephrogenesis. In humans, nephrogenesis is complete by week 36 of gestation, and 1 week after birth in mice (gestation is approximately 20 days in mice). The first glomeruli appears by week 9 of gestation in humans and at embryonic day 14 in mice [7]. Upon completion of nephrogenesis, each human kidney will have approximately one million nephrons [8], while in mice, total nephron count in each kidney is about 13,000 [9]. The process of

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kidney development and nephrogenesis has been extensively studied and described in numerous articles (including Chapter 1 of this book), and thus will not be described in depth here. However, a brief overview of kidney and nephron development will be described here.

To kick off pregnancy in humans, the sperm fertilizes the egg. Soon after fertilization, the egg forms the totipotent stem cell containing morula, which moves down the fallopian tube into the uterus, as seen in Fig. 4.1A. At day 5 post fertilization, the morula develops into the blastocyst. The blastocyst implants into the endometrium of the uterine wall 6–7 days after fertilization. The inner cell mass of the blastocyst contains the totipotent embryonic stem cells (referred to as the embryoblast) that will go on to eventually form the embryo. Depending on the exact location within the blastocyst, the embryonic stem cells of the inner cells mass will first go on to form either hypoblast or epiblast. Next, during gastrulation around 7–10 days post fertilization, the epiblast differentiates into the three primary germ layers: the ectoderm, mesoderm, and endoderm. Collectively, these germ layers give rise to all of the tissues and organs in the developing embryo. The mesoderm is the germ layer that gives rise to the kidneys.

As the three germ layers start forming different parts of the embryo, the pronephric duct (also referred to as the nephric or Wolffian duct) arises from the cranial portion of the intermediate mesoderm. The intermediate mesoderm is a narrow section of the mesoderm that is made up of multipotent stem cells. The nephric duct grows caudally along the length of the embryo's trunk [10]. Eventually, an outgrowth from the nephric duct emerges called the ureteric bud, which migrates toward and then invades the metanephric mesenchyme [7], as seen in Fig. 4.1B. The metanephric mesenchyme is a mass of pluripotent stem cells that contain embryonic stem cells [11]. The key to outgrowth and invasion of the ureteric bud into the metanephric mesenchymal is the release of the signaling ligand glial-derived neurotrophic factor (GDNF) by cells of the metanephric mesenchymal. GDNF activates RET receptor tyrosine kinase on the ureteric bud, which subsequently triggers its outgrowth invasion into the metanephric mesenchyme.

As the ureteric bud invades the metanephric mesenchyme, it releases signaling factors that cause the stem cells of the metanephric mesenchyme to condense into the cap mesenchyme around the tips of the ureteric bud. The cap mesenchyme releases more GDNF and other signaling factors that induce the ureteric bud to continue branching repeatedly. The multibranched ureteric bud eventually becomes the collecting ducts, renal pelvis, and ureter of the urinary system. Meanwhile, the ureteric bud secretes various factors that promote survival of the stem cells of the cap mesenchyme, including WNT proteins, fibroblast growth factors (FGFs), and leukemia inhibitory factor (LIF) [7]. The cap mesenchyme is primarily made up of stem cells that express the marker sine oculis-related homeobox 2 (SIX2). These $SIX2^+$ stem cells go on to form most of the nephron, including the proximal tubule, loop of Henle, and distal tubule. As the ureteric bud continues to branch and invade the metanephric mesenchyme, sections of $SIX2^+$ stem cells of the cap mesenchyme pack into spherical-shaped aggregates called renal vesicles on the underside of the ureteric bud branches, as seen in Fig. 4.1C. The cells of these renal vesicles undergo differentiation into epithelial cells as renal vesicles first elongate into comma-shaped bodies and then into further elongated and differentiated structures called S-shaped bodies. The distal end of the S-shape body fuses with the ureteric bud and becomes the distal tubule. The middle section of the S-shape body forms the loop of Henle, while the opposite head end of the S-shape body forms the Bowman's capsule and podocytes. Vascular cells migrate into the emerging Bowman capsule to form the glomerular capillaries. While the exact details of the subset of progenitor cells that form the vascular cells of the glomerular and peritubular cell networks have not been fully clarified, they are believed to arise from angioblasts within the metanephric mesenchyme [12, 13]. Another subset of multipotent progenitor cells of the metanephric mesenchyme that do not express SIX2, but instead express winged-fork head transcription factor 1 (FOXD1), go on to form the nonepithelial cells of the nephron, including glomerular mesangial cells, fibroblast, pericytes, vascular smooth muscle cells, and renin-secreting cells [7]. Once the ureteric bud finishes branching, nephrogenesis continues by a process called nephron arcade formation, in which multiple nephrons are induced to form and attach to the same ureteric bud tip [14]. The process of nephron arcade formation allows multiple nephrons to connect to the same collecting duct in the cortex [15].

Impaired nephrogenesis during maternal malnourishment

Maternal malnutrition during pregnancy promotes fetal programming, reduces nephron generation, and increases the risk for adult cardiovascular and kidney disease in the offspring [2, 16–18]. Such fetal programming induced by malnourishment can occur in offspring that are born at a normal birth weight [19, 20] or more often times at a low birth weight (LBW; < 2500 g is considered LBW as defined by the World Health Organization). The LBW baby frequently demonstrates a reduced total nephron endowment. Examination of human kidneys have shown a direct correlation between birth weight and nephron count estimating an increase of 257,426 glomeruli per kg increase

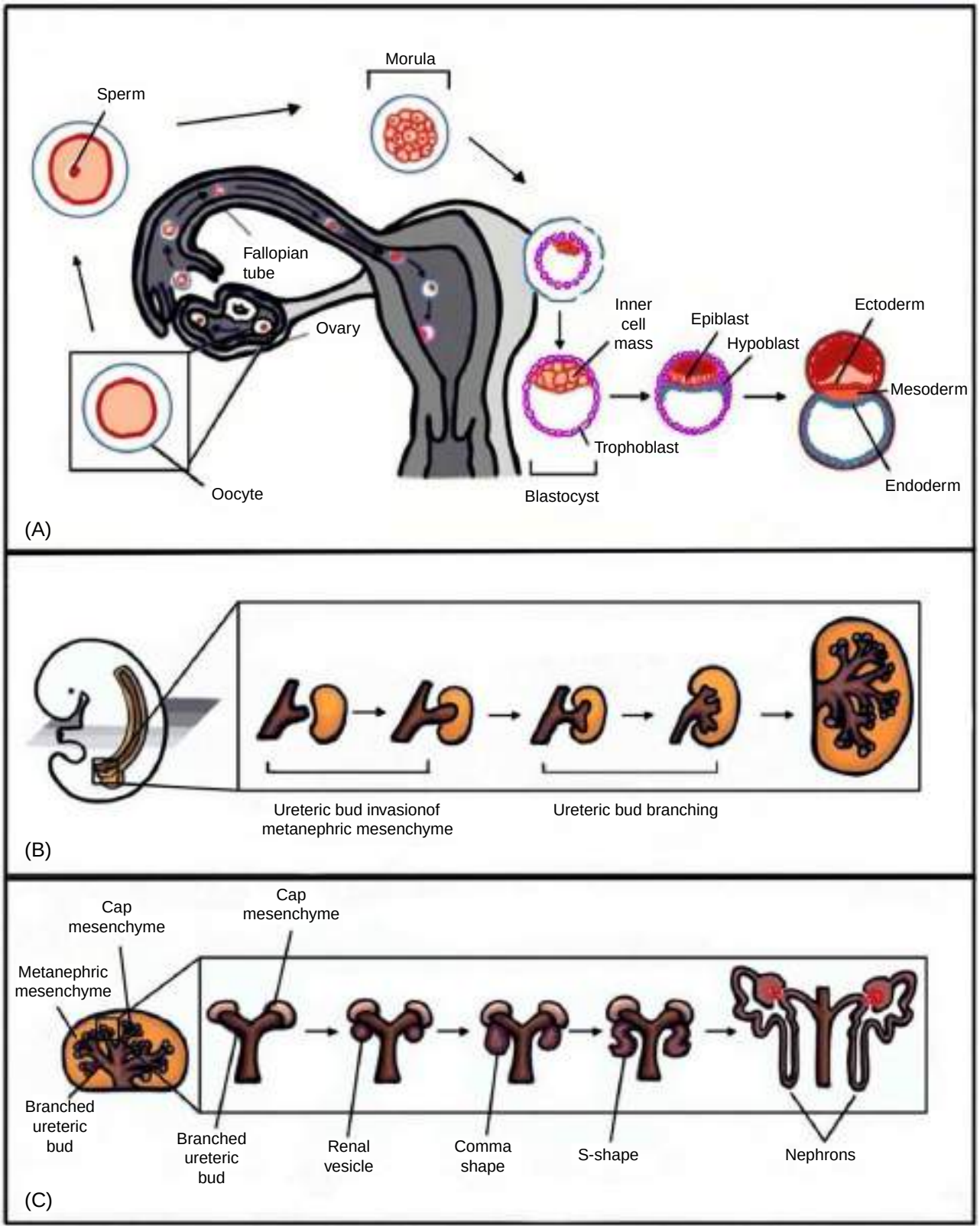

FIG. 4.1 Early fetal and kidney development. (A) After fertilization of the sperm, the egg develops into the morula and then the blastocyst. Embryonic stem cells of the inner cell mass of the blastocyst go on to create the three germ layers, of which the kidneys arise from the mesoderm. (B) At the early onset of kidney development, the nephric duct invades the stem cell ladened metanephric mesenchyme and then begins branching as the ureteric bud. (C) Stem cells condense into the cap mesenchyme around the tips of the branched ureteric bud. From the cap mesenchyme emerges renal vesicles, which go on to form C-shaped and the S-shaped bodies before finally becoming functional nephrons.

in birth weight [21]. The LBW baby also experiences a 70% increased risk for end-stage kidney failure as an adult [22, 23], including development of renal diseases, such as focal segmental glomerulosclerosis [24, 25], and potential hypertension [26–33]. LBW is associated with not only reduced nephron count but also reduced kidney size, reduced GFR, proteinuria, and an inverse correlation with salt sensitivity, which links it to the increase in blood pressure experienced by the LBW baby upon maturation into adulthood [22, 34, 35].

Various animal studies have examined the effects of maternal malnourishment during pregnancy on the offspring, including experiments that have restricted total maternal intake of total calories, protein, or individual micronutrients. In protein-restricted animal models, protein intake has been reduced from a normal intake of 18%–23% of total calories consumed, down to 5%–12% of total calories consumed. In rodent studies, a decrease in maternal protein intake during pregnancy has been shown to reduce total nephron count in offspring by approximately 15%–45%, with more severe protein deprivation leading to more significant reduction in overall nephron count [18, 28, 29, 36–40]. In these studies, it was observed that many of the generated glomeruli remained immature and not fully developed [40]. Animal studies also pointed out that female offspring are more resistant to the decline in renal nephron numbers, as compared with their male offspring counterparts, when protein deprivation is only mildly to moderately severe. However, as severity of protein depletion increases, both genders experience a comparable decline in nephron numbers [18]. Studies have also pointed out that protein deprivation in rodents during the second half of pregnancy (after about day 10 of gestation; gestation in a mouse last about 20 days, while in rats it last 20–23 days) when the kidneys are forming has a greater impact on kidney development and reduction in nephron numbers [18, 41]. However, one study demonstrated that protein restriction as early as the first 4 days after fertilization in rats causes blastocyst abnormalities, including reduced stem cell quantities in the inner mass of the early and late blastocyst apparently due to a slowed rate of cell proliferation [42]. Ultimately, the reduction in blastocyst stem cell numbers resulted in postnatal hypertension.

In addition to protein restriction, maternal calorie restriction during pregnancy similarly reduces nephron endowment in the offspring. In calorie-restricted animal models, global food restriction has varied in experiments to include reductions anywhere from 30% to 70% of normal intake. For example, restricting calorie intake in the pregnant mother by 50% causes a reduction in nephron count in the offspring by 30%–35% in various species. In studies, maternal calorie deprivation during the first half, second half, or all through pregnancy results in not only a decline in nephron number but also a reduction in GFR in the offspring at levels that are comparable between male and female offspring [43–45]. Calorie and protein restriction during pregnancy have also been found to impact not only total nephron count but also Na^+ excretion. Malnourishment impacts the renin-angiotensin system in the developing kidney by downregulating Ang II receptors and increasing Na^+ transport (including increased activity of Na^+/K^+ ATPase), effects that contribute to the offspring's salt sensitivity and development of hypertension [46–51].

Maternal malnourishment has been demonstrated to reduce nephron formation in the developing kidney by reducing critically needed stem cells, including those in the metanephric mesenchyme. Calorie and/or protein restriction has been shown to increase, by over twofold, apoptosis of mesenchymal stem cells of the metanephric mesenchyme in developing rodents [52, 53]. Similar decrease in nephron progenitor cells has been shown to impair nephrogenesis. In developing mice, genetic ablation of fetal nephron progenitor cells by 40% consequently reduced nephron endowment by 42% [52, 54]. This drop in progenitor cells reduced ureteric bud branching, presumably due to reduced GDNF signaling offered by the shrunken metanephric mesenchyme and reduced pool of $SIX2^+$ stem cells. Even gene mutations that disrupt $SIX2^+$ stem cells of metanephric mesenchyme inhibit the nephron formation, resulting in reduced total nephron numbers and smaller kidney size [55]. In a mouse model that employed a combined maternal calorie and protein-restricted diet during pregnancy, $SIX2^+$ stem cells in the metanephric mesenchyme of the developing kidney were reduced by 76% [52, 54], with increased apoptosis significantly contributing to their reduction. This finding is supported by the results of other studies on rats that observed maternal undernourishment during pregnancy alters apoptotic gene expression (Fas receptor and caspase 9) [56] in the embryo and increases apoptosis in the nephrogenic zone of the developing kidney [57]. Mesenchymal-to-epithelial signaling factors WNT9b and FGF8 are also downregulated in the embryonic kidney during maternal malnourishment, an event that further blunts nephron formation [52, 54]. These models highlight that the loss of critical stem cells during development consequently reduces nephrogenesis in the undernourished embryo.

Deficiencies in protein, carbohydrate, fat, and micronutrients impact kidney development not only directly but also indirectly by triggering the pregnant mother to alter her release of factors that detrimentally affect organogenesis. For instance, glucocorticoids are believed to impair kidney development. During malnourishment, activity of the hypothalamic-pituitary axis is enhanced, which stimulates the adrenal gland to increase glucocorticoid release in the pregnant mother [58]. Malnourishment also reduces the placental enzyme 11B-hydroxysteroid dehydrogenase (11B-HSD), which protects the developing embryo from excessively high and harmful levels of glucocorticoids [59].

Consequently, the developing kidney is exposed to abnormally high levels of glucocorticoids at times when nephrogenesis is ongoing. In rat experiments, introduction of the glucocorticoid dexamethasone to the developing kidney at similar concentrations inhibits ureteric bud branching and blunts nephrogenesis [60, 61]. Another example of a molecule whose release is stimulated by maternal malnourishment and impairs kidney development is the small protein fetuin-B. During malnourishment, bile release increases in the pregnant mother, which activates the farnesoid X receptor (FXR) in the placenta [62–68]. Activation of FXR turns up fetuin-B release, which then subsequently blunts nephrogenesis in the fetus. The upregulation of fetuin-B has been shown to decrease $SIX2^+$ stem cells by 50% in the developing kidney [62]. Fetuin-B increases NF-KB activity in $SIX2^+$ stem cells, leading to their apoptosis, along with their blunted proliferation due to upregulated p21 nuclear activity. Fetuin-B also increases reactive oxygen species (ROS) generation in the metanephric mesenchyme. While this increase in oxidative stress reduces the survival and self-renewal of renal progenitor cells, it is not clear if the oxidative stress is a cause or an effect.

Effects of macronutrient and micronutrient deficiency on kidney development

During pregnancy, a woman's overall need for consuming total energy is increased to meet maternal and fetal requirements. Additional energy intake is required during pregnancy for growth and maintenance of the developing baby, the placenta, and the mother's own tissues. While the fetus needs energy and the necessary building blocks for development, a lot of the increased energy intake is spent on the mother. During pregnancy, maternal basal metabolism increases due to increased metabolic activity of the cardiovascular, renal, and respiratory systems [69]. Increased energy demand is highest between week 10 and 30 of gestation, as this is when the bulk of fat is deposited in the pregnant mother's body [69]. While the fetus grows the most during the third trimester, the fetal demand for increased energy is offset by the near cessation of fat storage in the mother [70].

For optimum fetal growth and organ development to occur, not only are total energy requirements needed to be met by the mother but also appropriate intake of macronutrients (proteins, carbohydrates, and lipids/fats) and micronutrients (vitamins and minerals) are critically needed. This is especially true in regard to kidney development. Deficiencies in macro- and micronutrients during kidney development impair nephrogenesis and reduce nephron formation through mechanisms that reduce the stem cell pools that are critically needed to build important structures within the organ. Dietary deficiencies in nutrients adversely affect stem cells and reduce nephron formation by altering cell signaling, impacting epigenetic modifications, modulating gene expression, enhancing exposure to factors such as glucocorticoids and fetuin-B, and attenuating renal angiogenesis [71, 72]. Altered processing of microRNAs has also been demonstrated to reduce nephrogenesis. For example, when the activity of the microRNA activator Dicer is lost in renal progenitor cells during kidney development, there is an increase in apoptosis, a disrupted ureteric bud branching, and a premature termination of nephrogenesis resulting in reduced total nephrons [73]. In the following sections, the individual effects of macronutrients (proteins, lipids, fats, and fatty acids) and micronutrients (vitamins and minerals) are discussed. The exact effects of many of these molecules on kidney development and on critically needed embryonic and renal stem cells have yet to be completely elucidated.

Protein

Adequate protein intake during pregnancy is vital for healthy fetal development. Proteins provide the building blocks necessary for the fetus to form all of the necessary enzymes, antibodies, muscles, and collagen that the body needs [74]. Typically, while the pregnant mother may take in enough dietary protein, amino acids can get metabolized into energy for use by the mother, which can subsequently cause a protein deficiency for the developing baby in her womb. Obviously, poor nutritional intake can also lead to protein deficiency for the growing fetus. The majority of amino acids are supplied by the mother and moved from the maternal circulation into the fetus through placental amino acid transporters. However, maternal malnutrition during pregnancy downregulates placental amino acid transporters, which can further reduce the availability of key amino acids for the developing fetus to utilize [75].

While there are 20 recognized standard amino acids, there are many more nonstandard amino acids that occur through modifications to standard amino acids. Of the 20 amino acids, nine are considered essential, as the human body cannot synthesize them. These essential amino acids are phenylalanine, valine, threonine, tryptophan, methionine, leucine, lysine, and histidine. Information on the direct effects that essential and nonessential amino acid deficiency has on kidney development is relatively scarce. Interestingly, while protein deficiency can impair growth of the fetus, excessive protein intake can also adversely affect fetal development [76]. This highlights the need for balanced protein intake during pregnancy.

A key to the ability of amino acids to promote fetal growth is their actions on insulin release. Insulin is a major fetal growth factor that promotes anabolic effects in fetal tissues [77]. Leucine, lysine, arginine, alanine, and taurine have been shown to stimulate insulin secretion in the fetus [78–83]. Arginine also stimulates maternal growth hormone secretion, which alters maternal nutrient partitioning, thus directing more nutrients to go to the fetus instead of the mother [84]. Serving as a precursor for nitric oxide, arginine also enhances fetal growth by increasing uteroplacental perfusion due to its ability to increase local nitric oxide production [85]. Arginine, along with proline, further enhances fetal growth by enhancing placental growth through its promotion of polyamine synthesis [86].

Leucine enhances fetal growth through its effects on the mammalian target of rapamycin (mTOR) [87–89]. mTOR is a nutrient regulator, whose activation stimulates muscle protein synthesis in the fetus. Babies born at a LBW persistently have reduced muscle mass, indicating a reduction in leucine signaling. However, the exact mechanism involved in leucine regulation of mTOR is not known.

While diet is recognized as the ultimate influencer on the cellular environment and alterations in epigenetic patterns [90, 91], certain nutrients directly affect DNA and histone methylation modifications [92]. This especially includes the amino acid methionine, which impacts fetal growth and organ development through its regulation of epigenetic modifications. Methionine is converted in the body to *S*-adenosylmethionine (SAM), which serves as a vital methyl donor for many methylation reactions that involve DNA and histones [92]. Protein deficiency leads to methionine deficiency in the developing fetus, which significantly affects DNA methylation by reducing levels of SAM [92–94]. Dietary deficiency in methionine has been shown to induce profound DNA hypomethylation in a rodent model [95], an effect that consequently alters gene expression through epigenetic modifications. While the effects of such amino acid deficiency on fetal kidney development are not entirely clear, their deficiency is accepted to impair many critical aspects of nephrogenesis. Many of the vitamins and minerals that are subsequently discussed here also have similar effects on epigenetic regulation.

Lipids, fats, and fatty acids

The growing fetus requires the necessary building blocks for tissue and organ growth, which includes lipids. Therefore, adequate dietary intake of lipids is essential for the pregnant mother to meet the demands of the growing baby. Lipids are not only high-energy storage units but also function in various signaling pathways and are critical structural components of cell membranes [96, 97]. Lipids are an overall broad category of multiple hydrocarbon molecules that vary in structure and include fats, waxes, phospholipids, and steroids. Fats (typically known as triacylglycerols or triglycerides) are a subgroup of lipids that consist of two basic parts, a glycerol backbone and multiple fatty acid chains. Fatty acids in particular are important for normal fetal development. Fatty acid deficiency during development has been attributed to impaired brain development, cognitive impairment [98], neurodegenerative disease [98], cardiovascular disease, and altered renal tubular development in the offspring [99–102].

A deficiency in essential fatty acids leads to a decrease in linoleic (omega-6) and linolenic (omega-3) acids, both of which cannot be synthesized de novo and instead need to be obtained through diet. While both linoleic and linolenic acids are important membrane constituents, they also play a role in several other biological processes. For example, the essential polyunsaturated fatty acid linoleic acid is used in the biosynthesis of arachidonic acid, and thus it serves as a precursor for eicosanoids [74]. Eicosanoids function in the body, including the fetus and newborn, as important signaling molecules involved in many basic functions and reactions. These reactions and functions include secondary messaging, muscle relaxation, vascular constriction, immune function, inflammatory responses to injury and infection, and even pain [74, 102]. Conversely, the fatty acid linolenic acid is used to generate proinflammatory versions of eicosanoids. An equal balance of these two fatty acids, obtained by a balanced diet, creates a balance in the pro- and antiinflammatory signaling which allows for optimal immune function in the newborn [74]. Furthermore, an imbalance of essential fatty acids during development has been shown to reduce cholesterol and phospholipid generation [103], effects that would expectedly impact signaling pathways and cell membrane construction in the developing fetal kidney. In fact, fatty acid imbalance during kidney development has been shown to affect ATPase activity in the renal tubules, AngII expression, and potentially Na^+/volume handling and renal hemodynamics in young rats [99].

Vitamin A

While vitamin A is routinely introduced to the body through food intake, particularly intake of meats, the human body can also synthesize vitamin A from carotenes found in plants. Vitamin A is essential for growth, protein synthesis, and cell differentiation in the developing fetus [74]. The group of fat-soluble compounds known as vitamin A includes retinol, retinal, and retinoic acid [104]. In the human body, cells can reversibly generate the alcohol form of

vitamin A, which is retinol, by oxidative reduction of the aldehyde group in retinal. However, further conversion of retinol to retinoic acid is irreversible [104, 105].

Maternal vitamin A deficiency during pregnancy has been demonstrated to negatively impact kidney development and structure in the human and animal fetus [14, 106–111]. In rodent research, maternal vitamin A deficiency throughout pregnancy causes a reduction in total nephrons in offspring by as much as 20% [112, 113]. Meanwhile, human studies have suggested that maternal vitamin A deficiency during pregnancy causes renal hypoplasia and reduced renal volume in offspring, effects that appear to be associated with lowered maternal serum retinol concentration [107, 111]. In contrast, retinoic acid supplementation during maternal food restriction can overcome nephrogenesis impairment in the rat fetus [3]. Even in healthy pregnant animals (rats), injection of retinoic acid at midgestation yields offspring with as much as 21% more nephrons [113]. However, vitamin A should not be supplemented indiscriminately during pregnancy, as excessive vitamin A can also blunt nephrogenesis [114].

Animal studies suggest the impairment in nephron development, and nephrogenesis that accompanies vitamin A deficiency is mediated through three effects. First, retinoic acid serves as a critical ligand that activates RET receptors on the ureteric bud, thereby promoting ureteric bud branching. Without RET activation, there is a decline in ureteric bud branching and invasion of metanephric mesenchyme, which leads to a reduction in nephron formation and vascular growth [14, 112]. Second, vitamin A also appears to impact renal development through its antioxidative effects. The redox activity of retinol and retinal enables these two molecules to act as ideal antioxidants, thereby protecting stem cells from oxidative stress while effectively promoting stem cell survival and growth [104, 115, 116]. Third, vitamin A serves as a transcriptional regulator of embryonic and progenitor stem cells [115, 117]. Retinol, in particular, has been observed to maintain pluripotency and self-renewal of rodent embryonic stems cells (obtained from blastocysts) [118, 119] by enhancing NANOG and subsequently preventing embryonic stem cell differentiation [118]. This is in contrast to the effects of retinoic acid, which can serve as a transcription regulator [115, 117], a promoter of cell differentiation during embryogenesis, and an instructor of precursor cell commitment to separate cell fates during tissue patterning and organogenesis [105, 120–122]. Embryonic stem cells typically lack the enzymes that are required to metabolize retinol into retinoic acid [118]. However, retinoic acid can still make its way into these stem cells through its diffusion across the plasma membrane into the cytoplasm. Once inside the cell, retinoic acid activates its nuclear receptors, modulates intermediate transcription factors, and ultimately regulates over 500 genes, many of which are involved in stem cell differentiation [123]. A portion of the genes that retinoic acids impacts are involved in DNA methylation, histone acetylation, and histone methylation [104], explaining why retinoic acid increases DNA methylation and differentiation in embryonic stem cells [124, 125]. Retinoic acid also targets genes in embryonic stem cells that regulate proliferation and pluripotency, and therefore effectively suppresses pluripotent gene expression while promoting differentiation [126–128]. For example, retinoic acid can inhibit the canonical WNT signaling pathway, modulate AKT/mTOR signaling, and consequently alter the pluripotency of stem cells [104, 129]. The effects that vitamin A has on stem cells ultimately impact their ability to promote and maintain adequate nephrogenesis in the developing kidney.

Vitamin B

There are eight chemically distinct water-soluble compounds that are referred to as B vitamins. All eight B vitamins are essential and serve as either a cofactor (coenzyme) or an essential precursor for critical cellular metabolic processes. Various cellular metabolic pathways require one or more B vitamins, including glucose metabolism (glycolysis, pentose pathway, glycogenolysis, and gluconeogenesis), synthesis and degradation of fatty acids, amino acid degradation, the TCA cycle, and oxidative phosphorylation activity in the mitochondria [104]. The eight B vitamins include:

Vitamin B_1 (thiamine)
Vitamin B_2 (riboflavin)
Vitamin B_3 (niacin, nicotinamide, nicotinamide riboside)
Vitamin B_5 (pantothenic acid)
Vitamin B_6 (pyridoxine, pyridoxamine, pyridoxal)
Vitamin B_7 (biotin)
Vitamin B_9 (folic acid/folate)
Vitamin B_{12} (cobalamin)

One of the most studied B vitamins is B_9 (folate). Folate is the natural form of vitamin B_9 that is found in foods. Conversely, folic acid is man-made and is often used as a dietary supplement or is added to fortified foods during its processing for enhancement of micronutrients. Upon intake, the oxidized pteridine cyclic ring of folic acid is

quickly reduced in the human body to form folate [130]. The requirement for folate intake is greatly enhanced in the woman during pregnancy [74]. It is well documented that folate deficiency during pregnancy can lead to neural tube defects in the baby [131], most commonly spina bifida and anencephaly [74]. In addition to its impact on neurological development, folate also influences kidney development. For example, in a human study, maternal folate concentration in early pregnancy had a positive correlation with offspring kidney volume [132]. However, while folic acid supplementation may be a strategy for combating impaired kidney development in the embryo during maternal malnutrition, one study suggests that folic acid supplements used alone (and not part of a multivitamin supplement) can potentially cause congenital anomalies of the kidney and urinary tract [132, 133].

Folate, along with other B vitamins, are important for embryonic and kidney development due to their ability to form neurotransmitters, their promotion of cell growth and their crucial role in many methyl donation reactions, including production of thymine for DNA synthesis, polyamine synthesis, synthesis of methionine from homocysteine [74, 110, 134–137], and general gene methylation-associated epigenetic modulation [138]. For instance, vitamins B_6, B_9, and B_{12} synergistically affect DNA and histone methylation by serving as key coenzymes for the transfer of carbon molecules from amino acids for use in various biochemical reactions [139, 140]. In regard to folate, a carbon molecule is transferred from the amino acid serine to tetrahydrofolate (THF) to form 5,10-methylene-THF, which is needed for nucleotide synthesis [104]. The transfer of the carbon from serine to THF is mediated by a vitamin B_6-dependent enzyme. Folate also works with vitamin B_{12} in the methionine cycle. In this cycle, vitamin B_{12} is a coenzyme that promotes the conversion of homocysteine to methionine. This conversion occurs by the transfer of carbon from the folate cycle to homocysteine. Methionine is then converted to *S*-adenosylmethionine (SAM), which is a critical methyl donor for methylation of DNA, RNA, proteins, and lipids [141]. During the methionine cycle, the metabolism of homocysteine effectively lowers homocysteine concentrations [110, 132, 138]. Both observational and animal experimental studies have delineated a relationship between elevated homocysteine concentrations, glomerular damage, and a decrease in renal function [110, 138, 142]. Based on this relationship, it is concluded that increased homocysteine levels in the pregnant mother also impact renal development and function in the offspring [110].

The family of compounds that make up the class of vitamin B_3 are implicated in all cellular metabolic processes that utilize NAD or NADP, and thus are particularly important for the stem cells that are critical for renal development [104]. The three compounds that make up vitamin B_3 include niacin (nicotinic acid), nicotinamide (NAM), and nicotinamide riboside (NR) [104]. All these compounds are precursors for nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which are critical cofactors in a variety of stem cell metabolic reactions [104]. While nicotinamide riboside has been shown to increase mitochondrial recycling and cell survival in stem cells [143], the effects of nicotinamide on a variety of stem cell facets are even more widespread. For instance, nicotinamide has been reported to promote survival of embryonic stem cells [104], maintain organoid cultures of various cell types [144–146], promote expansion of progenitor cells [147], and even promote differentiation in certain cell types such as cardiomyocytes [148]. Although the exact nicotinamide-related molecular mechanisms responsible for such activities in stem cells have yet to be exactly identified, recent reports have emerged that highlight some of the mechanistic actions of nicotinamide. At high concentrations, nicotinamide influences epigenetic modifications and cell fate by inhibiting the deacetylase activity of sirtuins [149]. Nicotinamide also inhibits poly(ADP-ribose) polymerase (PARP), which is believed to improve cell survival by inhibiting apoptosis [104, 150, 151]. Also, at high concentrations, nicotinamide inhibits multiple kinases that are involved in cell survival and pluripotency [104]. For example, nicotinamide inhibits ROCK kinase-induced cell death. Furthermore, nicotinamide inhibits casein kinase 1, which leads to cessation of stem cell self-renewal and instead promotion of differentiation [152].

With such vast influence on various aspects of stem cells, it is logical to expect adverse effects on kidney development during vitamin B deficiency. Such logic is further supported by the necessity to include B vitamins in the basal culture medium of embryonic stem cells and embryonic kidney explants (which maintain their $SIX2^+$ stem cell populations and continue to undergo nephrogenesis in culture if maintained in vitamin B containing medium) [52, 153]. Inclusion of B vitamins in cell culture medium is necessary for maintaining stem cell growth and viability, including embryonic stem cells and renal $SIX2^+$ stem cells found in the developing kidney. Stem cells, including embryonic kidney explants, are typically maintained in Dulbecco's Modified Minimum Essential Medium (DMEM) combined with Ham's F12 medium (DMEM/F12). All eight essential B vitamins are found in DMEM/F12, which support long-term stem cell survival and self-renewal [104].

Vitamin C

Vitamin C, also known as ascorbic acid, is an essential nutrient that is involved in a variety of functions, such as tissue repair, neurotransmitter formation, enzymatic activity, immune system function, and antioxidant activity.

While vitamin C can be generated from glucose, in humans the enzyme responsible for the last step of conversion of glucose to vitamin C (L-gulonolactone oxidase) is inactive [154, 155]. Therefore, humans have to obtain vitamin C from food sources.

It is believed that vitamin C plays a role in mesoderm differentiation of embryonic stem cells [104, 156], potentially through epigenetic regulation. Vitamin C can modify DNA and histone methylation and thus trigger stem cell differentiation and reprogramming through epigenetic modulation [157–159]. Vitamin C has this effect on stem cell epigenetics by serving as an enzymatic cofactor for polyhydroxylates, along with DNA and histone demethylation [160]. Demethylation is catalyzed by Tet proteins [141, 161, 162], while histone demethylation is conducted by a group of histone demethylases called the Jumonji-C domain-containing family (JHDMs) [157, 163, 164]. The demethylase activity of Tet proteins and JHDMs are both vitamin C and Fe^{2+} dependent. Vitamin C works to keep Fe^{3+} reduced to Fe^{2+} (in a reaction that oxidizes vitamin C into dehydroascorbic acid) [165, 166], thus keeping the Fe^{2+} available for Tet and JHDM. By modulating the activity of these demethylases, and ultimately impacting epigenetic programming, vitamin C has been shown to increase the differentiation of various stem cells [167, 168], including both human and mouse embryonic stem cells [169–172]. This effect also includes the promotion of differentiation of hemangioblasts from embryonic stem cells [172], which may be an important contributor to vascular formation within the developing kidney.

Another key effect of vitamin C is its antioxidant effects, which impacts various biological pathways [104, 173]. During maternal malnutrition, the pregnant mother and developing fetal kidney experiences an increase in ROS that consequently disrupts renal $SIX2^+$ stem cells and nephrogenesis [62]. The antioxidant effects of vitamin C would be expected to offer protective effects on nephrogenesis by reducing detrimental ROS in the developing kidney. Vitamin C also promotes derivation and survival of stem cells, in both an oxidative redox dependent and independent fashion. For instance, oxidation of vitamin A to form dehydroascorbic acid (DHA) inhibits IκBα kinase β and subsequently modulates NF-κB signaling [174, 175]. Although the exact role of NF-κB activation has not been clarified in renal stem cells, particularly those involved in kidney development, it is believed to be related to a variety of stem cell processes including survival, proliferation, differentiation, and migration.

Vitamin C can also affect stem cells and kidney development through two other potential mechanisms as well. First, vitamin C controls extracellular matrix homeostasis [104, 176] through its effects on collagen protein folding and deposition [177–179]. By regulating the extracellular matrix, vitamin C has been shown to contribute to (human) embryonic stem cell attachment and survival [104, 177–179], factors crucial for metanephric patterning. Second, vitamin C suppresses hypoxia-inducible factor (HIFα) activity by reducing its transcription and simultaneously increasing its destruction by hydroxylase [104]. The loss of HIFα promotes mitochondrial activation, cell proliferation, and metabolism [180]. The role of HIF as a transcription factor is considered important to early fetal organ development. Therefore, vitamin C downregulation of HIFα would not theoretically be beneficial to the developing kidney early on, particularly in the early phase of metanephros formation, but instead perhaps plays a role in promoting differentiation of mesenchymal stem cells into glomerular and tubular structures in the cortex later in the developing kidney. This rational is supported by findings that show weak expression of HIFα in the cortex of the developing kidney when differentiation is high despite the low oxygen tension (and thus sustained hypoxic state) experienced by this fetal organ [181, 182].

Vitamin D

Several forms of vitamin D exist, including vitamin D_1, D_2, D_3, D_4, and D_5. All of the D vitamins are fat-soluble secosteroids (a steroid that has one of their steroid rings broken) that confer various biological effects, such as increasing intestinal absorption of calcium, magnesium, and phosphate [183]. The two major forms of vitamin D are considered to be D_2 (ergocalciferol) and D_3 (cholecalciferol). Collectively, the D_2 and D_3 forms are referred to as calciferol and are simply called vitamin D (without the number subscripts). Research has suggested that vitamin D_3 is more effective than vitamin D_2 at raising blood levels of vitamin D in humans. Thus, vitamin D_3 is the focus of this section, particularly since it is the natural form of vitamin D that the human body generates.

The active form of vitamin D is 1,25-dihydroxyvitamin D_3 (also known as calcitriol). However, to become activated, inactive vitamin D_3 must go through two hydroxylation steps, which occurs in two different organs. To start off, 7-dehydrocholesterol that is found in the lower layers of skin epidermis is converted to cholecalciferol (vitamin D_3) by ultraviolet light from the sun. Alternatively, cholecalciferol can be delivered to the body by food intake. Cholecalciferol undergoes its first hydroxylation in the liver to form 25-hydroxyvitamin D_3. Next, 25-hydroxyvitamin D_3 undergoes its second hydroxylation in the proximal tubule cells of the kidney to form 1, 25-dihydroxyvitamin D_3, which is the active form of vitamin D.

It is well known that sufficient vitamin D is required during fetal development to build and maintain strong bones and teeth [74]. Despite this, two studies have estimated that as many as 42%–54% of women of childbearing age in the United States have a vitamin D insufficiency (more specifically, a lack of 25-hydroxyvitamin D_3) [184, 185]. Regarding the adult kidney, a deficiency in vitamin D is associated with a twofold increased risk for the development of microalbuminuria [28]. However, it is less known the role that vitamin D has on kidney development.

In studies on rats, vitamin D deficiency during pregnancy did not alter kidney weight or kidney volume in the offspring, but instead unexpectedly increased nephrogenesis resulting in a significant 20% increase in total nephrons in the offspring's kidneys (although average renal corpuscle diameter in these offspring was decreased) [186]. Similarly, in mice, maternal vitamin D deficiency during pregnancy also resulted in a substantial increase in total nephrons, despite a decrease in overall glomerular diameter [187]. In the study on mice, it was observed that many glomeruli were not fully matured, implicating that although nephrogenesis is enhanced during vitamin D deficiency, full maturation and functionality of nephrons are impaired [187]. Human studies have thus far agreed with these rodent models. Human mothers deficient in 25-hydroxyvitamin D_3 give birth to children that have larger kidney volumes and an increase in GFR [46]. However, in contrast, a separate study in rats observed that vitamin D deficiency during pregnancy decreases kidney mass and impairs kidney function in the offspring [188]. Collectively, these animal and human studies indicate that vitamin D levels in the pregnant mother impact offspring kidney structure and function [46].

The mechanisms by which vitamin D effects kidney development and renal stem cells remain unclear [46]. Vitamin D is believed to be an important factor for cell proliferation, differentiation, and maturation [46, 189]. For instance, vitamin D has been shown to induce differentiation of mesenchymal stem cells along with stimulating adhesion and migration of endothelial progenitor cells (reviewed by YC Hou et al.) [190]. Furthermore, embryonic stem cells contain receptors for vitamin D (VDR) [191]. VDR is a member of the nuclear hormone family whose activation stimulates transcriptional regulation of various genes that have vitamin D response elements [192]. This suggests a potential role for vitamin D during the early stages of embryogenesis, which may include effects that protect embryonic stem cells critically needed for organogenesis [191]. Vitamin D has also been shown to increase the quantity of hematopoietic stem cells in the human umbilical cord during embryonic development [193], although the significance of this is unclear.

Vitamin E

Vitamin E is found in practically every tissue in the human body [194]. Vitamin E is made up of eight different fat-soluble compounds that include four tocopherols (in α, β, γ, and δ forms) and four tocotrienols (also in α, β, γ, and δ forms). The difference of each of these compounds is the number and position of methyl groups on the vitamin's chromanol ring [194–196]. The biological function of the vitamin E compounds varies to include antioxidant activity, regulation of kinase activity [197], and regulation of gene expression [198]. Although typically rare in the United States, vitamin E deficiency can occur. As opposed to a lack of dietary vitamin E intake, vitamin E deficiency is instead usually associated with an underlying intestinal problem that prevents vitamin E absorption.

Only a few studies have examined the effect of vitamin E, or more specifically α-tocopherol, on kidney development during maternal malnutrition. In one study on rats, maternal treatment with α-tocopherol during lactation significantly increased nephron endowment in offspring that had initially been born with a reduced nephron endowment due to maternal malnourishment during pregnancy [199]. In contrast, in a different study in pregnant rats, α-tocopherol treatment to undernourished mothers throughout pregnancy resulted in offspring that had impaired fetal renal development, which included retarded tubular development, upregulation of renal Na^+ transporting ATPases, and surprisingly an increase in renal superoxide production [200].

Despite the finding that α-tocopherol can stimulate renal superoxide production (which is most likely indirectly caused by α-tocopherol's effects on various signaling pathways), the antioxidant capacity of vitamin E is paramount to its protective effects on cells, including stem cells. In fact, vitamin E is recognized as a major antioxidant in human plasma [201]. All eight of the compounds that make up vitamin E contain a chromane double ring, with a hydroxyl group that can donate a hydrogen atom. This reducing capability of vitamin E effectively allows for its scavenging of free radicals, which subsequently protects cellular membranes and terminates polysaturated fatty acid oxidation [104]. For instance, as a fat-soluble compound, vitamin E can insert into the plasma membrane and prevent oxidative membranal damage, such as lipid peroxidation [201, 202]. Stem cells are especially sensitive to the effects of ROS, which can impact cellular signaling and differentiation [104]. Vitamin E can protect embryonic stem cells from ROS by its ability to neutralize free radicals [104]. For example, embryonic stem cell ROS-induced differentiation into vascular progenitor cells and endothelial cells is prevented by treatment with vitamin E [203, 204]. Outside of embryonic stem cells, vitamin E has been demonstrated to prevent ROS-induced apoptosis in mesenchymal stem cells via

the AKT pathway [205, 206], while also promoting the proliferation of dental pulp stem cells during oxidative stress [207]. Therefore, the subsequent protection of stem cells offered by the antioxidative activity of vitamin E would be expected to help maintain nephrogenesis.

During stress conditions, vitamin E can potentially prevent stem cell death and protect stem cell function by not only protecting against harmful oxidative stress but also through its influence on kinase-related signaling pathways [104]. Moreover, vitamin E modulates various signaling transduction pathways through its ability to regulate kinases, phosphatases, lipid mediators, and transcription factors [104, 208, 209]. For example, vitamin E targets protein kinase B (PKB/AKT) and either activates or inhibits (depending on the specific cell type) the PI3K/AKT pathway, which ultimately effects cell survival [206, 210–212]. Vitamin E, more specifically α-tocopherol, can also affect cell proliferation, adhesion, and gene expression through its inhibition of protein kinase C (PKC) [209, 213–217]. In addition, vitamin E regulates ERK [211], p38 MAPK [218], and WNT signaling [219], all of which are important signaling molecules for various pathways involved in proliferation, survival, and differentiation of renal stem cells during development. As a fat-soluble compound that can insert into the plasma membrane of stem cells, Vitamin E can also impact the activation of key kidney developmental signaling molecules through its interaction with, and structural and compositional modification of, the plasma membrane [208].

Zinc

Found in soil, seawater, and even in the atmosphere, the essential mineral zinc is the 24th most abundant element in the earth's crust. In the human body, zinc is the second most abundant trace metal, following iron. In the human body, zinc is a required cofactor for over 300 enzymes and over 1000 transcription factors [220, 221]. Included in the over 300 enzymes that require zinc are oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases [222, 223]. Zinc affects the catalytic activity and quaternary structure of many enzymes, while also promoting DNA binding to gene expression regulatory proteins [222]. Due to these effects, zinc plays a role in RNA and DNA metabolism, signal transduction, gene expression, and even regulation of apoptosis [224, 225].

Women require an increased intake of zinc during pregnancy, due to the necessity of zinc for normal embryogenesis, organogenesis, fetal growth, and development. Zinc deficiencies have been associated with growth retardation, delayed sexual maturation, increased susceptibility to infection, diarrhea, and impaired kidney development [225–228]. Regarding prenatal development, zinc sufficiency is extremely important in the first trimester when organs are first forming. At the onset of fetal development, zinc regulates chromatin structure and function, which subsequently impacts the expression of genes essential for embryogenesis [222, 229]. Despite the critical need for zinc, it is estimated that as much as 82% of pregnant women worldwide have an insufficiency in their zinc intake [230].

In humans, a zinc deficiency during development has been shown to negatively impact kidney development. In rat studies, maternal zinc deficiency during pregnancy blunts ureteric bud branching leading to reduced nephron numbers, nephron size, and GFR in the offspring [138, 226, 227]. The effect of maternal zinc deficiency in rats is also associated with increased oxidative damage and glomerular hypertrophy [226]. Similarly, postnatal rats subject to a zinc-deficient diet immediately following weaning (when developmental maturation is still undergoing) experience an increase in renal apoptosis along with a decrease in nephron count, glomerular capillary area, and GFR [228]. The effects of zinc deficiency in these animals is not restricted to only the kidneys but also the entire fetus due to zinc deficiency-related placental insufficiency. To this extent, zinc deficiency blunts trophoblast cell differentiation and implantation, leading to reduced fetal size [231].

On a cellular level, zinc is well recognized to play a critical role in cell division/replication, maturation, adhesion, and differentiation, leading to tissue and organ growth in the developing fetus [232]. These cellular effects are due to the role that zinc plays in numerous complex enzymatic and gene regulatory mechanisms, along with signal transduction pathways and hormonal regulation [232–234]. For example, zebrafish offspring born to zinc-deficient parents experience significantly reduced expression of key DNA methyltransferases (DNMT4 and DNMT6), which alters epigenetic progeny profiles [235]. Adequate levels of zinc have also been found to be crucial for maintenance of (mouse) embryonic stem cells [236]. In this regard, zinc is required for activation and signal transduction of the transcription factor STAT3 through its promotion of STAT3 phosphorylation [237]. The subsequent activation of STAT3 upregulates genes crucial to maintain pluripotency and self-renewal of embryonic stem cells [238].

Zinc also appears to be involved in the promotion of cellular defenses against oxidative stress and associated tissue damage in the developing kidney, as observed in an animal study in which zinc deficiency in the pregnant mother led to enhanced renal oxidative stress in the developing offspring kidney [227]. In this animal study, elevated oxidative stress caused lipid peroxidation and apoptosis of cortical cells (including cells of the glomeruli) in the kidneys of the offspring [227]. Furthermore, maternal zinc deficiency effectively decreases renal catalase activity, glutathione

peroxidase activity, and glutathione levels in the offspring kidney, all of which are key players in the prevention of oxidative damage [227]. It is believed that these crucial antioxidant and oxidoreductase systems are downregulated due to the reduced signal transduction and transcriptional factor regulation caused by zinc deficiency. Overall, the loss of protection against oxidative stress that leads to renal tissue damage and a decrease in cortical cells (and presumably stem cells of the metanephric mesenchyme) would significantly blunt nephrogenesis.

Iron

Iron is by mass the most abundant element found on Earth and the most abundant trace element in the human body. Iron is essential to maintain life in humans. A major role for iron in the human body is its incorporation into heme proteins, including hemoglobin, myoglobin, and cytochrome. Iron's incorporation into these compounds allows them to transport and store gases, build enzymes, and transfer electrons. While many foods are a vastly rich source of iron, especially certain meats, iron deficiency is the most common nutritional deficiency in the world [239–241]. Quite common is iron deficiency in the pregnant mother, which can lead to iron-deficient anemia, subsequent reduced oxygen delivery to the growing fetus, and adverse effects on fetal organogenesis [242, 243].

While the effects of iron deficiency have been extensively studied in the brain (in which iron deficiency causes adverse neurological effects), studies that report the effects of low iron levels on renal development are sparse. In a study on rats, maternal iron deficiency during pregnancy was associated with a reduced total glomerular number in the offspring [244, 245]. However, the mechanistic reasons for the reduced nephron endowment in the study are not clear. In addition to its critical role in gas transport and tissue oxygenation, iron is also a crucial structural component for many redox enzymes that are involved in cellular respiration and oxidation/reduction reactions, further highlighting the importance of iron in maintaining normal fetal development and growth [74]. While iron has multiple redox states, the most common oxidative states of iron are Fe^{2+} and Fe^{3+}. This redox characteristic of iron makes it a key cofactor for enzymes (such as catalase) involved in oxidation-reduction reactions that occur in stem cells during metabolism that protect these cells against adverse ROS signaling and damage [246]. Altered enzymatic activation induced by iron deficiency can modulate downstream signaling pathways that govern stem cell regulation, and perhaps kidney development. For instance, studies on pluripotent stem cells showed that iron depletion causes downregulation of NANOG, followed by a consequential sharp decline in the self-renal of (human) pluripotent stem cells and their spontaneous and nonspecific differentiation [247]. The downregulation of NANOG is attributed to increased activity of lipoprotein-associated phospholipase A2, leading to an increase in lysophosphatidylcholine generation and subsequent epigenetic modifications that suppress NANOG expression [247]. This same effect on renal stem cells would expectedly impair nephrogenesis during kidney development.

Other minerals

While we have already discussed two minerals, iron and zinc, there are another 14 essential minerals. These other 14 essential minerals include calcium, phosphorus, potassium, sulfur, sodium, chloride, magnesium, copper, manganese, iodine, selenium, molybdenum, chromium, and fluoride. The nutritional need for many of these minerals increases significantly in women during pregnancy, particularly iodine, magnesium, and selenium [74]. While it is unclear the exact role that each of these specific minerals has on nephrogenesis and kidney development, their effects on cells (including renal stem cells) and biological processes are to some extent known.

Iodine: Iodine is a required factor for the generation of thyroid hormones thyroxine and triiodothyronine, both of which regulate growth, and therefore could potentially affect kidney development. During pregnancy, the dietary intake of iodine goes up for three main reasons, including (1) increased thyroid activity, (2) an increase in circulating thyroxine binding globulin, which decreases levels of free circulating hormone, and (3) the deiodinase activity of the placenta [248]. While iodine deficiency leads to hypothyroidism in general, its deficiency during pregnancy is known to increase the risk of mental retardation, along with impaired growth, hearing, and speech in the newborn offspring [74]. The effects of iodine deficiency on fetal kidney development have not been defined.

Magnesium: The human body contains over 300 enzymes that require magnesium for the stimulation of their catalytic activity. Enzymes that require magnesium include all of the enzymes that either use or synthesize ATP, along with enzymes that synthesize DNA and RNA from nucleotides [249]. Magnesium deficiency can lead to cardiovascular, neuromuscular, and metabolic dysfunction [250]. Magnesium deficiency is common in women in the United States and has been shown in pregnant women to increase the risk of miscarriage, fetal growth retardation, and preterm delivery [251]. While the exact effects of magnesium deficiency on fetal kidney development have not

been fully elucidated, it is associated with a considerable increased risk for sudden infant death syndrome (SIDS) [74]. Considering the critical role that magnesium plays in ATP, DNA, and RNA synthesis, magnesium deficiency would be expected to impact various aspects of kidney development.

Selenium: Selenium is an integral component in the structure of the amino acid derivatives selenocysteine and selenomethionine. Selenium and these selenoproteins function as vitally important cofactors involved in the reduction of two major thiol/disulfide-based antioxidant enzymes, including five isoforms of glutathione peroxidase [252] and three isoforms of thioredoxin reductase [253]. By keeping these enzymes reduced, the antioxidant capabilities of the glutathione and thioredoxin systems are maintained. Selenomethionine also has antioxidant activity by itself and can separately recycle glutathione. Due to these effects on critical antioxidant systems, selenium would be expected to potentially play an important role in the prevention of oxidative damage in the developing fetal kidney. Not surprisingly, selenium deficiency is associated with a decrease in glutathione peroxidase activity in women [254]. The role of selenium is not limited to its antioxidant activity though. The thyroid gland, and any cell in the human body affected by thyroid hormone, also use selenium. Cells use selenium as a cofactor for thyroid hormone deiodinases. These deiodinases are peroxidase enzymes that regulate activation/deactivation of thyroid hormones and their metabolites [255]. However, it is not clear if the effect that selenium has on thyroid hormones impacts kidney development.

Copper: Copper plays an important role in anaerobic respiration and ATP synthesis in the mitochondria of all cells, including renal stem cells. Due to copper's relatively easy conversion between Cu(I) and Cu(II), copper is found in cytochrome *c* oxidase where it modulates electron and oxygen transport [256]. Multimeric cytochrome *c* oxidase (also known as complex IV of the mitochondrial respiratory chain) is the last protein in oxidative phosphorylation. In cytochrome *c* oxidase, O_2 is bound to copper and iron molecules. This conformation allows cytochrome *c* to transfer electrons to the O_2 molecule, thereby releasing the chemical energy of oxygen to generate ATP. Copper is also present in many superoxide dismutases, and thus plays a role in antioxidant systems and degradation of superoxides [257]. Neonatal copper deficiency has been shown to decrease superoxide activity in newborn rats [258]. The decrease in superoxide activity is associated with excessive lipid damage, protein damage, and cell death (reviewed by Keen et al.), effects that would inevitably impair kidney development [258]. Copper deficiency can also lead to a reduction in lysyl oxidase activity. Reduction of lysyl oxidase activity decreases collagen and elastin cross linking, which detrimentally affects organ development [259].

Conclusion

Maternal malnourishment during pregnancy impairs nephrogenesis and kidney development, leaving the resulting baby at an incredibly high risk for the occurrence of kidney and cardiovascular disease throughout life. The impairment occurs not only in babies that are born of low birth weight due to maternal malnourishment but also in babies born at a normal birth weight. Maternal malnourishment results in deficiencies in macronutrients and micronutrients, which consequently leads to the impairment in kidney development. Altered cell signaling, growth factor regulation, gene expression, oxidative stress, and especially altered epigenetic modifications appear to be the major culprits behind the impairment in nephrogenesis that is promoted by malnourishment, as summarized in Fig. 4.2. While these changes impact different mechanistic pathways vital to normal development, a decrease in critically needed stem cells that generate nephrons and kidney structures is at the root of the problem. Lack of vitamins, minerals, and amino acids reduce the self-renewal and survival capacity of embryonic and renal stem cells. This detrimentally reduces the size of the stem cell pool available for nephron building during nephrogenesis. While each vitamin, mineral, and even amino acid has a distinctive role in embryonic development and organogenesis, there also appears a tremendous amount of overlap in their effects as well. Many of these nutrients commonly affect epigenetic modifications and gene expression, while also offering on some level protection against oxidative stress. In the developing embryonic kidney, these effects are heavily felt. As the severity of malnourishment intensifies, the decline in total nephrons formed becomes more drastic as kidney underdevelopment grows worse. While supplementation during pregnancy with individual vitamins (such as retinoic acid, folate, iron, and zinc) can improve certain aspects of kidney development, supplementation strategies should instead include a more complete balanced mix of combined nutrients, including vitamins and minerals in addition to macronutrients (protein, lipids, fasts, and fatty acid). These macronutrients are the structural building blocks needed for developing cells, tissues, and organs, and thus are just as critical for normal kidney development as are micronutrients (vitamins and minerals). However, these nutrients all appear to have a defined concentration/dose at which their effects are optimal. Too much of many of these nutrients can adversely affect kidney development as well.

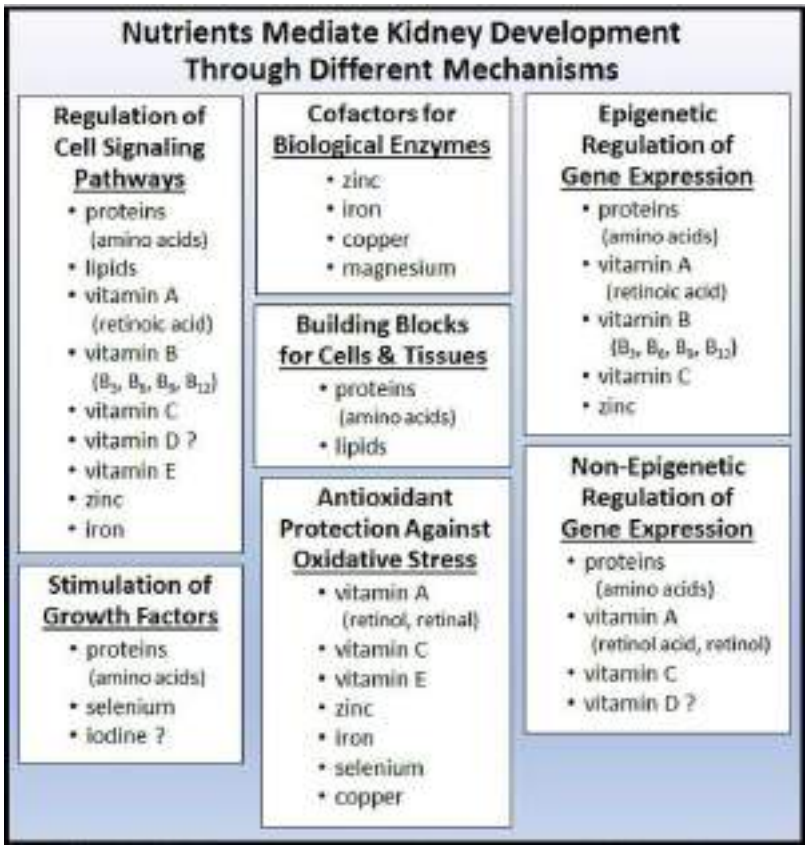

FIG. 4.2 Nutrient deficiency impacts kidney development through different mechanisms. The figure lists the pathways by which specific nutrients affect kidney development. A deficiency in these nutrients impacts their respective mechanistic pathway and subsequently impairs kidney development, usually involving a reduction in stem cells at some point throughout the process of fetal and kidney development.

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PART II

Modulators of regeneration

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CHAPTER

5

Endogenous antiinflammatory and proresolving lipid mediators in renal disease

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Introduction

Inflammation and its timely resolution is a physiological process that is critical to effective homeostasis promoting pathogen clearance and/or tissue repair and regeneration [1]. It is now appreciated that insidious, unresolved inflammation is a key driver of numerous pathologies including diseases of the vasculature, organ fibrosis, and organ failure in response to both chronic and acute insults [2, 3]. In the context of the pathogenesis of kidney disease, inflammation drives adverse events in the glomerulus, vasculature, tubules, and interstitium. Therapies targeting inflammation and the immune system play crucial roles in the management of many kidney diseases and have done so for many years. However, we remain challenged in clinical nephrology to find equipoise between efficacy and the toxicity of many of these treatments, which aim to halt the immune system activity. Attempts to uniformly "block" or slow down the activity of the immune system are associated with immune compromise and metabolic complications, which are of particular relevance in the context of management of chronic conditions. In recent decades, we have refined our appreciation of the complex nature of inflammation and come to understand the dynamic mechanisms which underpin effective resolution are critical to the avoidance of ongoing tissue injury and propagation of chronic kidney disease [4]. Identification of the molecular signals and distinct processes that blunt inflammation and drive its resolution have generated insights into potential novel therapeutic opportunities [5, 6]. In this chapter, we will review the mechanisms of action of lipid mediators that drive the resolution of inflammation and recent work that aims to translate our knowledge of biology into effective and tractable therapeutics in kidney disease. An overview of some of these effects is highlighted in Fig. 5.1.

Lipid mediators of inflammation

The early part of the innate immune response is effected by polymorphonuclear neutrophils (PMNs), and we now appreciate that the termination of this initial response reflects both the dissipation of the inciting stimulus and the generation of specific mediators which mediate endothelial activation and PMN extravasation, apoptosis, and clearance from an inflammatory focus by macrophages. The role of lipid mediators in regulating the onset, progression, and eventual resolution of these processes is established [2, 3]. As we will discuss, under physiological conditions as the inflammatory response evolves and resolves, there is a switch in lipid mediator biosynthesis from the archetypal proinflammatory signals including specific leukotrienes and prostaglandins to specialized, proresolving lipid mediators (SPMs) [20]. SPMs are derived from polyunsaturated fatty acids and are important in ensuring that the destructive inflammatory response, necessary at the time of initiating insult/injury, does not persist [21], thereby averting insidious low-grade inflammation characteristic of many noncommunicable chronic diseases, such as atherosclerosis, obesity, cancer and indeed, chronic kidney disease (CKD) [4]. Initial observations of responses to SPMs focused

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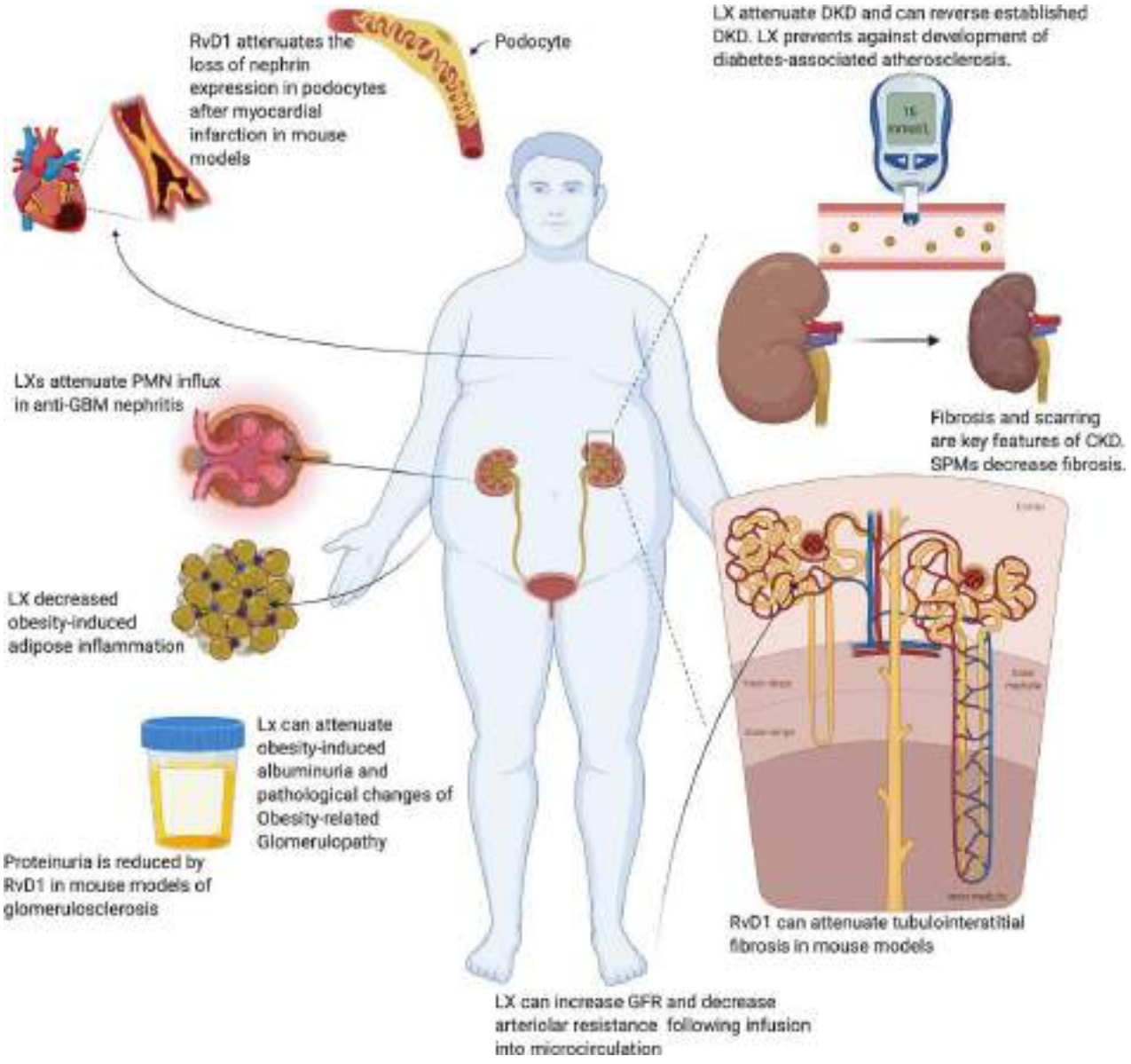
RvD1 attenuates the loss of nephrin expression in podocytes after myocardial infarction in mouse models
Podocyte
LX attenuate DKD and can reverse established DKD. LX prevents against development of diabetes-associated atherosclerosis.
LXs attenuate PMN influx in anti-GBM nephritis
Fibrosis and scarring are key features of CKD. SPMs decrease fibrosis.
LX decreased obesity-induced adipose inflammation
Lx can attenuate obesity-induced albuminuria and pathological changes of Obesity-related Glomerulopathy
Proteinuria is reduced by RvD1 in mouse models of glomerulosclerosis
RvD1 can attenuate tubulointerstitial fibrosis in mouse models
LX can increase GFR and decrease arteriolar resistance following infusion into microcirculation

FIG. 5.1 Overview of effects of SPMs in kidney disease. Proresolving lipid mediators have been shown to have many effects in models of kidney disease. PMN influx is attenuated in models of acute glomerulonephritis, and subsequent fibrosis is diminished [7–9]. Inflammation associated with obesity is decreased and obesity-related glomerulopathy, including proteinuria, improved [10–14]. Established changes associated with diabetic kidney disease (DKD) can be successfully reversed [11]. The characteristic fibrosis seen in Chronic Kidney Disease (CKD) can be ameliorated [15–17]. Renal plasma flow and eGFR can be improved [18]. Podocyte injury and loss following myocardial infarction can be attenuated by treatment with RvD1 [19]. *Figure produced with the aid of BioRender.com.*

on their roles at limiting PMN extravasation and promoting efficient efferocytosis of apoptotic PMN by macrophages at an inflammatory focus [22, 23]. As we will discuss, more recent developments have highlighted a role for SPMs in fibrosuppression, regeneration, and repair [2, 4].

The first class of lipid mediators recognized to evoke antiinflammatory and proresolving responses were the arachidonate-derived lipoxins [2]. Subsequent investigations of lipid mediator biosynthesis in models of self-limiting inflammation revealed the existence of several other classes of SPMs including the resolvins [24], maresins [25], and protectins [26]. In more recent years, we have come to appreciate the potential impact of ineffective SPM responses in driving inflammation [27, 28]. Currently, efforts are being made to harness the proresolving properties of these lipid mediators and synthetic analogs as therapeutics, for acute and chronic diseases, in nephrology and other spheres [5, 10, 11, 29–31].

The local generation, activity, and inactivation of lipid mediators are critical to their role in modulating inflammatory responses allowing for appropriate containment of processes which, if widely disseminated, could be catastrophic. Tissue injury, which may be initiated by trauma, surgery, or microbial invasion, results in the release of arachidonate (AA)-derived eisocanoids [2, 3]. AA can be metabolized by cyclooxygenases (COXs), lipoxygenases (LOXs), or cytochrome P450–dependent mixed function oxidases [3].

The enzymatic generation of prostaglandins and leukotrienes is well established in response to both pathogens and sterile injury. PGs and LTs regulate blood flow, vasodilatation, and permeability changes facilitating leukocyte adhesion, diapedesis, and recruitment (e.g., reviewed in 3). Prostaglandins (PGE2 and PGD2) also signal the termination of inflammation by upregulation of expression of enzymes that generate additional mediators as discussed.

Lipoxins (LXs), an acronym for lipoxygenase interaction products, are typically generated by transcellular metabolism at an inflammatory focus. Lipoxin A4 and Lipoxin B4 are the principal LXs formed in vertebrates [32]. Aspirin acetylation of COX-2 generates metabolites which can in turn be converted via 5-lipoxygenase to 15-*epi* LXA4 (sometimes termed aspirin-triggered LX; ATL) [33]. Statin-induced modification of COX-2 also generates intermediates in 15-epiLX synthesis. The generation of epi-LXs has been proposed to account for some of the beneficial, unanticipated antiinflammatory responses to statins and to aspirin [34]. In the context of acute inflammation, LXs promote the resolution of inflammation by limiting further PMN recruitment: modulating endothelial adhesion and PMN transmigration. LXs stimulate macrophage efferocytosis of apoptotic PMN at the sites of inflammation [22, 23]. This is a critical step in promoting resolution and repair. Failure to "clear up" apoptotic leukocytes results in the accumulation of necrotic, toxic debris that perpetuates injury [35].

The resolvins [24] are SPMs derived from precursors eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the major n-3 fatty acids, also widely known as the omega-3 polyunsaturated fatty acids (PUFAs). These compounds are biosynthesized in resolving exudates and possess potent actions, controlling the duration and resolution of inflammation and are designated *E*-series (eicosapentaoic acid-derived) and D-series (docosahexanoic acid-derived) resolvins, respectively. DHA-derived mediators in neural tissue have shown protection in models of stroke and are designated neuroprotectins [36]. Maresins (*ma*crophage mediators in *re*solving *in*flammation) are DHA-derived SPMs generated in macrophages. Peptide-conjugated maresins have noteworthy roles in tissue regeneration [37]. The balance of responses to cysteinyl leukotrienes versus peptide-conjugated maresins is a key determinant of lung pathology and may provide insights into variable therapeutic responses to leukotriene antagonism in asthma [38, 39]. The work of Serhan and colleagues has stressed that the biosynthesis of resolvins and protectins is stereochemically controlled via enzymatic reactions that give rise to chemical mediators that elicit potent bioactions and require precise stereochemistry to activate specific receptors in contrast to the autooxidation products obtained from DHA, EPA, or other PUFAs, which may also arise in tissues. Such autooxidation products are complex mixtures that can serve as biomarkers of oxidative stress. Much of the work that has detected SPM generation has focused on lipidomic analysis of exudate in models of self-limiting inflammation and reveals highly conserved pathways [40].

Importantly, the generation of SPMs (lipoxins and resolvins) has been detected in a self-limiting inflammatory response in humans (UV-killed *E. coli*-induced skin blister model) and injection of a cocktail of SPMs at the peak of inflammation (i.e., maximal PMN accumulation) was demonstrated to accelerate the time to resolution (i.e., PMN clearance) [41]. A detailed description of the pathways of biosynthesis, stereochemistry, and challenges of detection and quantification of endogenously generated SPMs is beyond the scope of this chapter and readers are referred to numerous excellent recent reviews (e.g., 3).

Receptor activation by SPMs

The SPMs exert their proresolving actions through distinct G-protein coupled receptors (GPCRs). To date, four GPCRs have been identified as receptors for lipoxin A4 and the D- and *E*-series of resolvins, namely ALX/FPR2, RVD1/GPR32, RVD2/GPR18, and RVE1/ChemR23 (see Fig. 5.2).

LXA4 is a high-affinity ligand for a GPCR-designated FPR2/ALX. The receptor is a member of the formyl peptide receptor family (FPR) and is proposed to play a pivotal role in initiating the resolution of inflammation [6, 42]. The human FPR family is clustered on Chromosome 19 and encodes a family of three Class A GPCRs [43]. FPRs play a critical role in innate immune responses through recognition of pathogen-derived stimuli (Pathogen-Associated Molecular Patterns (PAMPs)) and sterile provocation (Damage-Associated Molecular Patterns (DAMPs)). FPR2/ALX is highly expressed in myeloid cells and also in cells of diverse origin, including endothelia, epithelia, smooth muscle cells, chondrocytes, and fibroblasts [35]. FPR2/ALX displays many intriguing

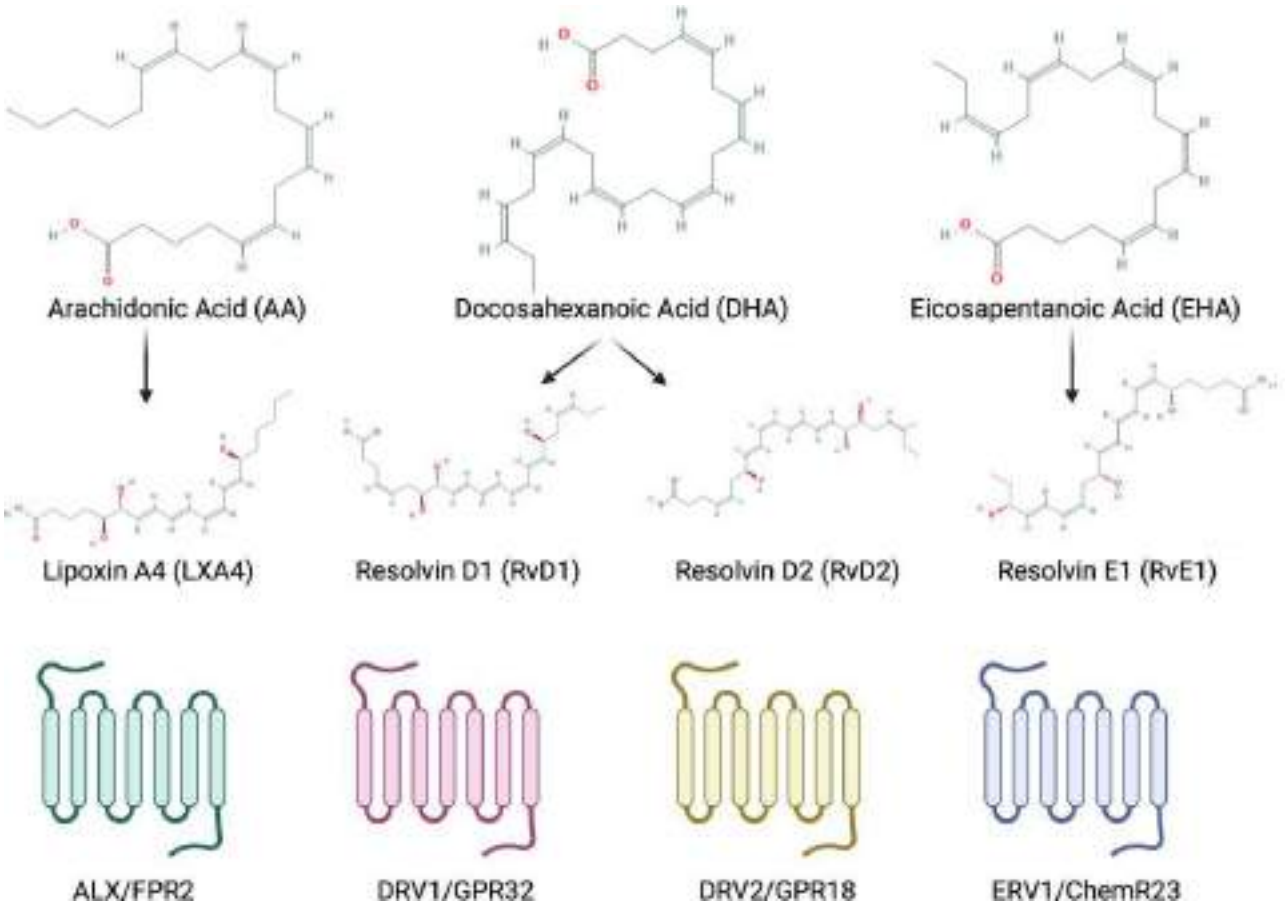

FIG. 5.2 Specialized proresolving mediators (SPMs) and their receptors.

physiological and pharmacological responses as reflected by its activation by diverse ligands including proteins, peptides, and lipids, which may provoke antiinflammatory responses typified by lipoxins or annexin-derived peptides or proinflammatory responses when activated by ligands such as the acute phase protein serum amyloid A [44]. Recent evidence highlights a role for dimerization of the FPR family in dictating proinflammatory versus antiinflammatory responses [45] and the impact of biased agonism on cellular responses [46].

Genetic deletion or overexpression of FPR2/ALX highlights the role of the receptor in regulating acute inflammation [42, 47, 48]. A genetic polymorphism [rs11666254] associated with decreased FPR2/ALX expression is associated with compromised immune responses [49]. It is interesting to note that LXA4 also acts as an agonist of the orphan receptor GPR32 [50] (as there is no murine homolog of GPR32, genetic ablation has not been informative in generating experimental models]). LXA4 is a functional antagonist of cysLTD4 receptors [51] and acts as an allosteric enhancer of cannabinoid CB1 receptor [52].

In addition to the direct activation of GPCRs, LXs can regulate the activation of receptor tyrosine kinases, including EGF, PDGF [53, 54], and VEGF [55, 56] receptors, by mechanisms which may include the activation of phosphatases to regulate the phosphorylation status of specific tyrosine residues [57]. In renal epithelia, LX induction of the let-7 family of miRNAs targets expression of the TGFb R1 receptor, therefore regulating serine threonine receptor kinases [30].

RvD1 binds to the lipoxin A4/annexin-A1 receptor/formyl peptide receptor 2 (FPR2/ALX) as well as to GPR32 [50], which is also activated by additional RVDs (RVD3, RVD5 [58, 59]). RVs can inhibit the activation of transient receptor potential channels and therefore attenuate inflammatory pain [60]. RvE1 and chemerin (Chem), an endogenous peptide agonist, bind to the Chem receptor 23 (ChemR23; [61]). ChemR23 shares 36% sequence homology with FPR2/ALX. RVE1 also binds the LTB4 receptor BLT1 on PMN [61, 62]. RvD2 activates GPR18 and the effects of RvD2 to limit PMN trafficking are not observed in GPR18 −/− mice [63].

Renal inflammation, resolution, and fibrosis

Inflammation is a feature of most renal pathology, and severe acute or chronic renal inflammation may lead to glomerulosclerosis, tubular atrophy, damage to renal vasculature fibrosis, and eventual renal failure [64, 65]. The pathogenesis is complex, but irrespective of the initial stimulus, infiltration of neutrophils followed by monocytes and lymphocytes is observed. Resolution of renal inflammation involves the removal of apoptotic leukocytes and a change in the cytokine milieu from proinflammatory to antiinflammatory coupled to restoration of vascular tone. There is growing evidence for a role of antiinflammatory, proresolution lipid mediators in these processes as in other organs and diseases. As we will discuss, such mediators target multiple aspects of the inflammatory cascade: inhibiting activation of leukocytes and trafficking from the vasculature, promoting clearance of apoptotic cells, inhibiting the perpetuation of chemotactic signals for additional leukocyte recruitment, and contributing to the maintenance of epithelial and mesangial cell integrity, resulting in the maintenance of kidney functionality and a net antifibrotic phenotype. The key cellular events and targets are highlighted in Fig. 5.3.

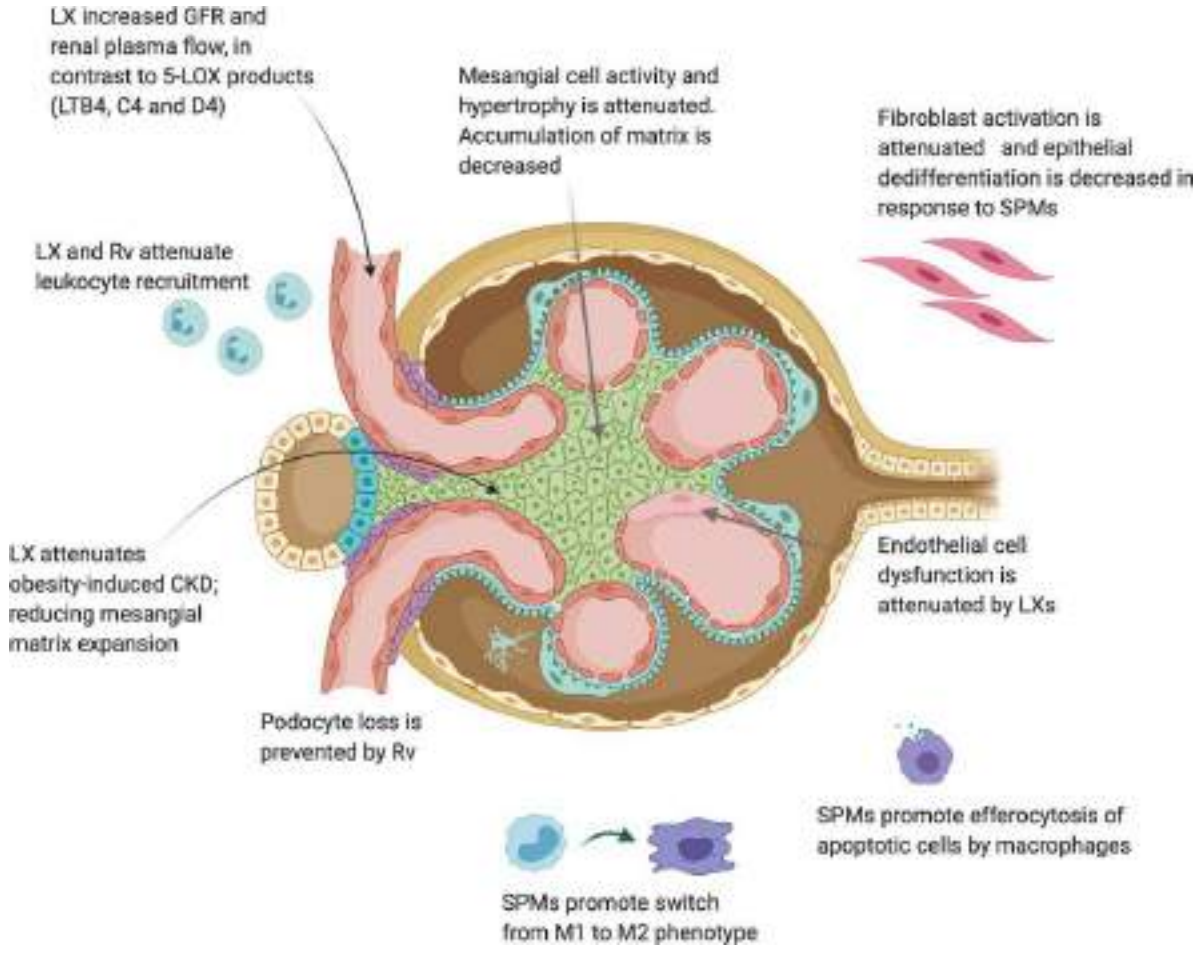
LX increased GFR and renal plasma flow, in contrast to 5-LOX products (LTB4, C4 and D4)
Mesangial cell activity and hypertrophy is attenuated. Accumulation of matrix is decreased
Fibroblast activation is attenuated and epithelial dedifferentiation is decreased in response to SPMs
LX and Rv attenuate leukocyte recruitment
LX attenuates obesity-induced CKD; reducing mesangial matrix expansion
Endothelial cell dysfunction is attenuated by LXs
Podocyte loss is prevented by Rv
SPMs promote efferocytosis of apoptotic cells by macrophages
SPMs promote switch from M1 to M2 phenotype

FIG. 5.3 Cellular targets and actions of SPMs. Glomerular scarring and fibrosis are characteristic features of chronic kidney disease. Common histological findings include mesangial expansion, collagen deposition, basement membrane thickening, podocyte loss, tubular epithelial damage, accumulation of activated myofibroblasts and matrix, and influx of inflammatory cells. SPMs can target key steps in renal inflammation and fibrosis. LXA4 can decrease arteriolar resistance and increase GFR [18]. Leukocyte recruitment and activation can be attenuated by SPMs [22, 66, 67]. Dedifferentiation of epithelial cells and cytokine production can be decreased [30, 67, 68]. Endothelial cell activation [55, 69], fibroblast to myofibroblast activation [29, 70], and podocyte effacement [71] can all be attenuated by SPMs. A switch from M1 to M2 macrophage phenotype is promoted in the presence of SPMs [10, 23, 29], while mesangial cell activation and accumulation of mesangial matrix are decreased [16, 17, 53, 54]. *Figure produced with the aid of BioRender.com.*

Acute glomerulonephritis represents an important cause of acute kidney injury (AKI) that if unresolved may result in chronic kidney disease (CKD). The current mainstay therapy for this disease is immunosuppression. Long-term maintenance treatment is often required with a risk of serious adverse effects. In this regard, novel therapeutic strategies that would switch the cellular response from inflammation in favor of resolution mimicking and/or amplifying endogenous homeostatic drives are of great interest [5, 65].

The first compelling experimental evidence for a role for LXs as counterregulators in renal physiology came from investigations of the glomerular microcirculation, where it was observed that, in contrast to 5-LOX products (LTB4, C4, and D4) generated during the acute inflammatory phase, LXA4 increased glomerular filtration rate (GFR) and plasma flow and decreased arteriolar resistance subsequent to intrarenal infusion of LXA4 into the microcirculation. Furthermore, direct antagonism of LTD4-induced hemodynamic changes by LXA4 was also reported [18]. In the concanavalin A ferritin model of immune complex glomerulonephritis, treatment of rat neutrophils ex vivo with LXA4 reduced their subsequent trafficking into inflamed glomeruli [7]. The potential importance of transcellular generation of LXA4 as a modulator of renal inflammation was demonstrated in a model of nephritis in p-selectin-deficient and wild-type mice, where p-selectin deficiency inhibited platelet-endothelial and neutrophil-endothelial interaction. The p-selectin mice exhibited exaggerated renal PMN influx and decreased LXA4 production relative to wild-type mice [7]. Analogously, manipulation of renal 15-LOX expression by intrarenal delivery of 15-LOX cDNA in a rat model of glomerulonephritis was associated with increased LXA4 production by the kidney and maintenance of renal function relative to nontransfected controls [8].

Modulation by LXs of key inflammatory mediators, such as NF-κB, IL-6, and proliferative pathways including Akt and Jak-STAT, indicates that LX may not only provide an effective therapy limiting tissue damage arising from inflammation but also modulate the process of renal fibrosis subsequent to glomerulonephritis. Acute poststreptococcal glomerulonephritis typifies a self-resolving condition and it is noteworthy that maximal LTB4 levels have been observed at the peak of the inflammatory response which dissipated, whereas LXA4 levels persisted during resolution and remained elevated. In this setting, increased LXA4 was shown not only in leukocytes but also in the glomeruli, indicating both a systemic and a local proresolution effect [9].

Mesangial cell proliferation and activation is a frequent feature of renal inflammation. At a cellular level, LXA4 has been shown to modulate the proliferation of mesangial cells induced by cytokines and growth factors that have been implicated not only in glomerulonephritis but in other disorders such as the development of tubulointerstitial fibrosis [15–17]. LXA4 inhibits LTD4 binding to human mesangial cells. In addition to acting as a partial agonist at cysLT receptors, LXA4 binds to ALX/FPR2 in human mesangial cells. Intriguingly, LXA4 inhibits PDGF-, EGF-, and connective tissue growth factor (CTGF)-stimulated proliferation of mesangial cells, independent of cysLT-mediated receptor transactivation [17, 53, 54, 57]. Mesangial cell fibronectin and collagen production are critical components of glomerulosclerosis. LXA4 counteracts PDGF-induced fibrosis-related gene expression in mesangial cells and renal epithelia [30, 54], suggesting that LXA4 is an endogenous regulator of fibrosis and that mimetics may act as potential antifibrotic agents preventing the progression of renal disease. This hypothesis is further enhanced by evidence that LXA4 modulates TNF-α-induced proliferation and cytokine release as well as CTGF-mediated release of fractalkine, MCP-1, and RANTES [17]. Administration of synthetic aspirin-triggered LX analog (ATLa), before ischemia, resulted in significant functional and morphological protection and attenuated chemokine and cytokine responses [66]. Using a transcriptomic approach to explore the events that underlie this protective effect, it was found that treatment with the ATLa, before injury, modified the expression of many differentially expressed pathogenic mediators, including cytokines, growth factors, adhesion molecules, and proteases [68]. This modulation of transcriptomic responses included many genes expressed by renal parenchymal cells and was not merely reflective of a reduced renal leukocyte recruitment. Of particular interest is the induction of suppressor of cytokine signaling-1 (SOCS-1) and SOCS-2 proteins and kidney injury molecule-1 (KIM-1) expression in this setting [72]. SOCS proteins have been identified as inducible feedback inhibitors of cytokine receptors and have been shown to be of crucial importance for the limitation of inflammatory responses, modulating dendritic cell and macrophage function [73], and represent an important target for promoting resolution of inflammation. Kidney injury molecule-1 (KIM-1) has come to prominence as a more reliable biomarker than serum creatinine in both human and animal models of AKI [72]. KIM-1 confers on epithelial cells the ability to recognize and phagocytose dead cells that are present in the postischemic kidney and contribute to the obstruction of the tubular lumen characteristic of AKI. The importance of clearing apoptotic debris in promoting resolution of inflammation has been outlined earlier, and further exploration of potential LX induction of KIM-1 may have important implications for the development of therapeutics for AKI [74]. In a model of acute antiglomerular basement membrane (anti-GBM) nephritis, an analog of LX was also shown to be protective, significantly limiting PMN influx [75].

The potential roles of resolvins and protectins in murine renal ischemia reperfusion injury (IRI) have been described. Results from Duffield and colleagues established the important principle that resolvins (RvD1) and protectins (PD1) are

formed during ischemic injury [67]. Bilateral renal ischemia followed by 24h reperfusion triggers endogenous biosynthesis and release of the precursor DHA into circulation. Postischemic kidneys also generate protectins, PD1, and to a lesser extent, RvD1 and RvD3. Administration of exogenous RvDs or PD1 before bilateral renal ischemia was associated with reduced leukocytes (PMN and monocyte influx) and an attenuation of increased plasma creatinine. Treatment with RvD1 or synthetic RvD1 after ischemic injury also demonstrated a protective effect. In contrast, PD1 did not show significant protection after the onset of ischemic injury. Histological examination of tissue 48h following ischemic injury demonstrated maintenance of tubular integrity in animals pretreated with RvD and PD1. In contrast to what has been described for LX in the maintenance of epithelial integrity [30], the authors discounted a direct effect of the agents on epithelia, as they were unable to show attenuation of oxidant-induced stress to cultured renal epithelia. However, this does not necessarily restrict the effects to leukocyte recruitment or activation, as endothelial and mesangial cells may also be affected. In these experiments, in addition to significantly reducing monocyte (CD68-positive) recruitment, both RvD and Protectin D1 inhibited LPS-stimulated TNF-α production from rat bone marrow-derived macrophages. Given the evidence that postinflammatory scarring of the kidney may play a major role in the development of CKD, the authors investigated whether RvD and PD1 altered renal fibrosis by investigating collagen deposition 15 days post-IRI. In animals treated with RvD but not in those treated with PD1, there was a significant decrease in fibrosis [67]. Hassan and Gronert used dietary manipulation to prime endogenous generation of PD1 in murine IRI. In animals fed n-3 PUFAs versus n-6 PUFAs, there was a marked attenuation in their resistance to renal IRI (plasma creatinine, PMN influx, and inflammatory cytokine production) that was attributed to PD1 biosynthesis and HO-1 upregulation. Systemic treatment with PD1 generated similar effects to those observed with endogenously generated PD1 [76]. Aspirin-triggered RvD1 has also demonstrated renal protection in endotoxin-induced AKI [77]. A role for T regs in RvD-induced protection in renal IRI has been proposed [78].

The overwhelming experimental evidence that SPMs promote the resolution of inflammation is supportive of a role in preempting fibrotic responses. More intriguing are data directly demonstrating the capacity of SPMs to attenuate preexisting fibrosis.

Matrix generation and accumulation in the tubulointerstitium and glomerulus can be influenced by SPM through several mechanisms, both direct and indirect. These include attenuation of responses of mesangial cells to growth factors such as EGF and PDGF via ALX/FPR2-mediated receptor tyrosine kinase activation [53, 54, 57]. PDGF induces reactive oxygen species generation in mesangial cells and this is attenuated by LXA_4 [57]. LXs elicit distinct, receptor-mediated responses in cellular targets of fibrosis including epithelia, fibroblasts, macrophages, and glomerular mesangial cells [35].

Epithelial cell injury in response to TGF-β1 can perpetuate a fibrotic response through generation of soluble mediators. LXA_4 attenuates TGF-β1-induced responses such as Jag-1 and CTGF expression [29, 30]. LXA_4 also inhibits TGF-β1–induced fibronectin and thrombospondin expression, key fibrotic responses. A detailed investigation of the underlying mechanism revealed distinct patterns of micro RNA (miRNA) expression in response to TGF-β1 that were modified by LXA_4. Of particular interest was LXA_4-mediated upregulation of the let-7 family of miRNAs [30]. Predicted targets of let-7 include the TGFβ-R1, a component of the TGF-β1 receptor dimer. Indeed, LX-induced upregulation of let-7 was associated with decreased expression of TGF-βR1 and an attenuation of responses to TGF-β1, including thrombospondin, Jag-1, and CTGF expression. These investigations suggested that the ability of LXs to sustain let-7 expression could reflect an important mechanism of action of LXs in maintaining renal tissue homeostasis. Importantly, in biopsy tissue from humans with CKD, let-7 target genes including the prototypic fibrotic molecules collagens and thrombospondin are upregulated, reflecting decreased levels of let-7 in the kidney [30].

let-7 delivery has subsequently been shown to suppress fibrosis in a model of kidney injury [79]. In unilateral ureteric obstruction (UUO), an experimental model of renal fibrosis, LXs modulated fibrotic responses [29]. Renal gene and protein expression, collagen deposition, macrophage infiltration, and apoptosis were analyzed. These data demonstrated that LXs attenuate collagen deposition and renal apoptosis, and also shift the inflammatory milieu toward resolution, inhibiting TNF-α and IFN-γ expression, while stimulating proresolving IL-10. LXs attenuated UUO-induced activation of MAP kinases, Akt, and Smads in injured kidneys, suggesting effects on both canonical and noncanonical TGF-β1 signaling. LXs blunted responses of cultured renal fibroblasts to TGF-β1 including proliferation, Smad activation, and gene expression [29].

Subsequent to these investigations, Qu and colleagues described the impact of RvE1 and D1 on renal fibrosis [70]. Using the UUO model they reported a direct effect of RvE1 in attenuating fibrosis when given before surgical obstruction and daily thereafter for the duration of the study. RvE1 treatment was associated with attenuation of fibrotic responses including collagen IV deposition, α-smooth muscle actin (α-SMA) positivity (a marker of myofibroblast activation), myofibroblast production, and PDGF production in the obstructed kidney. In this model, endothelial dysfunction associated with decreased eNOS3 was detected, and this was attenuated by RvD1 [69]. RvD1 suppressed Smad-3 phosphorylation in the linker region, Smad-3-JNK complex formation, and collagen I promoter activity.

RvD1 attenuates experimental glomerulosclerosis: reducing proteinuria, glomerulosclerosis, tubulointerstitial fibrosis, it modified macrophage recruitment and prevented synaptopodin loss characteristic of retaining podocyte integrity [12]. Here, adriamycin-induced nephropathy increased acetylation of 14-3-3-b protein was associated with a loss of synaptopodin and this was attenuated by RvD1. In vitro studies using cultured mouse podocytes demonstrated that RvD1 prevented TNF-α-induced synaptopodin loss. The authors proposed that the functional protection observed with RvD1 in Adriamycin-treated animals reflects protection from podocyte damage. This may be of particular importance, given the functional significance of podocyte loss in numerous kidney diseases. The protective effects of RvD1 may be mediated through ALX/FPR2, which is expressed in the mouse kidneys and in podocytes and was attributed to preventing acetylation of 14-3-3-b protein, thereby facilitating persistence of the degradation-resistant 14-3-3-b-synaptopodin protein complex [71].

The effects of LXs on obesity-associated pathologies have been investigated in a murine model [10]. As anticipated, obesity caused distinct pathologies including impaired glucose tolerance, adipose inflammation, fatty liver, and CKD. LXs decreased obesity-induced adipose inflammation, attenuating TNF-α expression and M1 $CD11c^+$ macrophages (MΦs), while restoring M2 $CD206^+$ MΦs. Given the role of CD206 + macrophages in the clearance of matrix debris, it is tempting to speculate that this may be one of the mechanisms through which SPM can subvert fibrotic responses. LXs attenuated obesity-induced CKD, reducing glomerular expansion, mesangial matrix, and oxidative stress. LXs also attenuated liver damage, as discussed below. LXs did not affect high fat diet– (HFD–) induced renal or hepatic MΦs, suggesting that protection occurred via attenuation of adipose inflammation. As reported for LXs, RvD1 similarly skews adipose macrophages toward an M2 phenotype in HFD-induced obesity [12] and also in obese diabetic mice [80]. Deficits in adipose SPMs have been reported in obesity in human and murine tissue, consistent with accelerated enzymatic breakdown [13]. Indeed, stabilization of omega-3-derived antiinflammatory epoxides using an inhibitor of soluble epoxide hydrolase has proven beneficial in a murine model of obesity [14]. In transgenic mice, overexpression of LXA4 generating enzymes in adipose resulted in higher LX levels in adipose browning of white adipose, protection against diet-induced obesity, insulin resistance, and hepatic steatosis [81].

Diabetic kidney disease (DKD) is the leading cause of CKD and kidney failure requiring replacement therapy. To date, DKD has been considered an intractable chronic complication of diabetes affecting those with both Type 1 and Type 2 diabetes. The mainstay of treatment for the past two decades has been control of the RAAS in an effort to slow disease progression. Recent advances [82] include using inhibitors of the proximal tubule sodium-glucose transporter to facilitate glycemic control (e.g., canagliflozin) in T2 diabetes and which have also shown beneficial effects on incident and progressive renal and cardiovascular endpoints [83]. DKD is characterized by glomerulosclerosis reflecting matrix accumulation, podocyte loss, and tubulointerstitial fibrosis in response to chronic metabolic and hemodynamic stresses that drive inflammatory responses [84, 85]. Antiinflammatory strategies have been proposed in the context of DKD including targeting of adhesion molecules and chemokines (e.g., VCAM-1, CCL-2) cytokines and intracellular signaling pathways including JAK-STAT with baricitinib and the antioxidant response driven by Keap-Nrf-2. There is evidence for genetic susceptibility to DKD and several of the polymorphisms identified are associated with inflammatory pathways [86]. Of particular interest given the evidence that LXs modulate EGF receptor activation [57] are SNPs in the ErbB4 gene [87]. ErbB4 is the heterodimerization partner of EGFR and upregulation of ErbB4 increases the risk of DKD [88].

Using the streptozotocin-treated ApoE −/− murine model of experimental diabetic complications, Brennan and colleagues demonstrated the efficacy of LXs to prevent both the development of DKD and to reverse established DKD [11]. LX treatment did not alter diabetes-induced hyperglycemia but LXs significantly reduced diabetes-induced albuminuria and glomerulosclerosis. Renal gene expression analysis revealed that these changes were associated with suppression of the Egr-1 pathway. Several egr-1 target genes are upregulated in human DKD [11]. In addition to responses seen in the kidney, LXs suppressed the development of diabetes-induced atherosclerosis and reversed plaque burden [31]. LXs were shown to inhibit VSMC proliferation and monocyte-endothelial adhesion, key features in the development of atherosclerosis. Similar responses have been seen in other SPMs [89]. The inflammatory responses of human atheromatous plaque ex vivo are blunted by LXs. [31].

From biology to therapy?

Conventional therapies for inflammation rely on antiinflammatory strategies. Implicit in this approach is the compromise of host defense [90]. An alternative therapeutic paradigm is to promote the resolution of inflammation by mimicking the biology of agonists of resolution typified by SPMs. It is especially important to note

that responses to SPMs consistent with resolving inflammation do not compromise normal host defense. On the contrary, there are numerous demonstrations of enhanced protection from pathogens in response to SPMs [91]. LXs promote the production of antibacterial peptides from epithelia such as bactericidal permeability increasing protein [92]. In experimental models of polymicrobial sepsis, treatment with SPMs (LXs, resolvins, protectins, and maresins) has been demonstrated to attenuate organ injury and enhance bacterial clearance and survival [93–96]. The role of FPR2/ALX in these processes is highlighted by the observation of enhanced severity of response to sepsis-induced injury in an FPR2/ALX$^{-/-}$ model [48]. Lipid emulsions that enhance the generation of endogenous SPMs have shown protection in sterile peritonitis and polymicrobial sepsis [97]. The enhanced pathogen clearance observed in response to sepsis may reflect enhanced phagocytic capacity of macrophages in response to SPMs [95]. In mouse models of *E. coli* infection, addition of exogenous resolvins reportedly lowered the threshold for antibiotic efficacy [58].

Tissue repair and regeneration in the absence of scarring are the final steps in complete resolution of inflammation. The role of SPMs in these processes is increasingly appreciated [37]. D-series resolvins stimulate arteriogenic revascularization in murine hind limb ischemia [98]. In models of muscle damage, exogenous addition of RvD1 limits the degree and duration of the inflammatory response, enhances regenerating myofiber growth, and improves recovery of muscle strength, polarizes intramuscular macrophages to a regenerative phenotype and stimulates local muscle stem cell response [99]. Intestinal epithelial wound healing has been demonstrated in response to RvE1-stimulated proliferation and migration [100]. Wound healing in diabetes is enhanced by RvD1 and RvE1 via the promotion of macrophage-mediated apoptotic cell clearance and reepithelialization [101].

There are several challenges to exploiting the therapeutic potential of SPMs. Many of the endogenously generated mediators are unsuitable as drugs per se being chemically and/or biologically unstable and synthesis is challenging. To circumvent this, several generations of synthetic SPMs and agonists of SPM receptors have been generated.

First-generation synthetic LXA4 analogs were designed in 1995–98 by Serhan, Petasis, and colleagues to minimize metabolism of the molecule [102]. These relatively stable pharmacological agents provided powerful tools to explore lipoxin functions in vivo. Among these, pharmacokinetic analysis of aspirin-triggered LX analogs (ATLa), ω-oxidation as a novel route for LXA4 metabolism, prompting the development of second-generation 3-oxa-LXA4 analogs with improved pharmacokinetic disposition [103] and potency and efficacy comparable to that of ATLa in diverse animal models after topical, intravenous, or oral delivery [103]. Whereas earlier synthetic analogs focused on generating molecules resistant to dehydrogenase activity at C15 and potential ω-oxidation at C20, subsequent efforts have focused on replacing the triene core. Lipoxin analogs featuring a benzo-fused ring system have been designed and proved to be as efficacious as native LXA4 in a series of in vitro and in vivo studies [104, 105]. More recent LX mimetics and FPR2 agonists generated include dimethyl imidazole–containing compounds [106] and compounds which are protective in models of myocardial ischemia reperfusion and heart failure [107–109]. A series of stable analogs of Rvs have been successfully synthesized including the addition of ω-terminal p-fluorophenoxyl to RvE1 and RvD1, which blocks metabolic inactivation without attenuating the antiinflammatory proresolving activities [110].

To date, clinical trials to investigate the efficacy of SPMs have been limited. In a randomized controlled trial, topical LX analogs reduced the severity of eczema in a study of 60 infants with efficacy comparable to those of topical corticosteroids [111]. Inhaled 5(*S*),6(*R*)-LXA$_4$ methyl ester and BML-111, an FPR2 agonist, displayed efficacy and safety in a pilot study of asthmatic children with acute moderate episodes, although efficacy was reduced compared to that of the standard of care (salbutamol) [112]. In models of renal fibrosis, obesity, and diabetic complications, LX analogs generated by replacement of the triene core with a benzene ring were potent and effective mimics of the activity of LXA4 [10, 11, 29, 31]. An alternative benzo-LX is currently under investigation as a topical oral rinse treatment of gingivitis (clinicaltrials.gov; NCT02342691). The evidence of accelerated resolution in the human skin blister model [41] provides a potential screen of putative proresolution therapies.

Conclusion

Inflammation is a central part of renal pathology, and resolution of renal inflammation is vital for the return to normal kidney function following injury. Evidence to date briefly summarized in this chapter indicates that a new era has emerged in the appreciation of inflammation and its progression to resolution or fibrosis. The SPMs play an active role(s) in controlling and programming resolution of inflammation by serving as agonists on their respective receptors, stimulating endogenous antiinflammatory, and proresolving pathways. Given their potency

and identification of targets for which they are endogenous regulators as defined through multiomics systems–based analysis [113], mimicry or amplification of their bioactions represents attractive targets for novel therapeutics. The increased recognition of ALX/FPR2 as a "targetable" GPCR to promote the resolution of inflammation in response to agonists with appropriate pharmacodynamic and pharmacokinetic profiles suggests that translation of these findings cannot be far off [114].

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CHAPTER

6

T cells in kidney injury and regeneration

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Introduction

Inflammation is an important event in the cascade of humoral and cellular responses that ultimately result in apoptosis and/or necrosis early following acute kidney injury [1–6]. Following ischemia–reperfusion injury (IRI), mononuclear phagocytes including dendritic cells (DCs), which normally reside in the interstitial microenvironment to maintain homeostasis, are activated by danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) [7, 8]. In the presence of PAMPs or DAMPs, these mononuclear phagocytes initiate inflammation through the activation of the innate immune system (which is comprised of neutrophils, monocytes/macrophages, DCs, and innate-like T cells—natural killer [NK] cells, natural killer T [NKT] cells, etc.) (Fig. 6.1). T-lymphocytes are traditionally thought to participate in adaptive immunity, however early studies demonstrated the importance of T-lymphocytes in early innate immunity [9–11]. Emerging technologies have aided studies of leukocyte subsets including lymphocytes and have shed new light on the role of immune cells in acute kidney injury and regeneration.

Leukocyte subsets can be detected and quantitated by flow cytometry analysis and ImageStream® analysis [12, 13]. Quantitative immunofluorescence microscopy demonstrated that flow cytometry–based assays highly underestimate kidney tissue–resident leukocytes (>20-fold) [14]. Multicolor histocytometry was used for visualizing and quantitating phenotypically complex cell populations directly with three-dimensional (3D) images [15, 16], which allow us to study in the future the localization of kidney tissue–resident immune cells and their interaction with the local tissue microenvironment. Parabiosis studies, characterized by a shared blood supply between two surgically connected animals, have been used to assess potential resident immune cell subpopulation replenishment from bloodborne cells and for investigating resident and recruited immune cell subpopulations in kidney diseases [17, 18]. Single-cell RNA sequencing (scRNA-seq) and gene-editing technologies are increasingly being applied to reveal cellular heterogeneity in mouse and human kidney diseases. The data revealed their transformative potential for the investigation of kidney physiology and disease [19–21]. Advances in CRISPR-Cas9 gene editing have provided encouraging new concepts and therapeutic approaches through protective gene programming in kidney diseases [22, 23]. Lastly, understanding specific pathways that regulate kidney-resident lymphocytes, which have increasingly been uncovered through novel technologies, is critically important for designing new strategies for blocking acute and chronic kidney diseases. The following sections will summarize the complex role of T cells in acute kidney injury and regeneration through studies that have employed new as well as conventional technologies.

Conventional T cells and resident kidney T cells

Naïve T cells develop and mature in the thymus before entering the circulation and are maintained in a quiescent state. Upon antigen (Ag) stimulation, they differentiate into effector T cells (Teff) and long-lived memory T cells [24]. T cells are classically divided into distinct subsets that express αβ or γδ T cell receptors (TCRs). αβ TCR responds to foreign peptide antigens that are bound to MHC class I or II proteins. αβ T cells include $CD4^+$ and $CD8^+$ T cells

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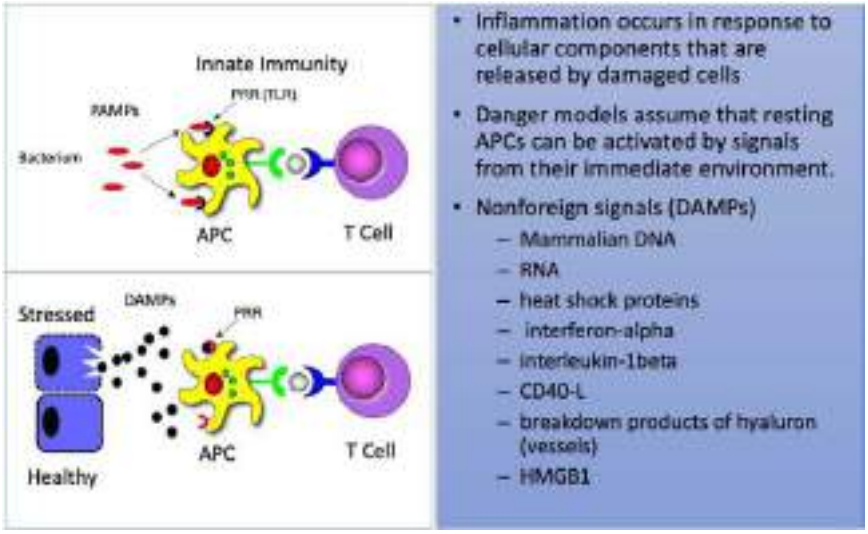

FIG. 6.1 Activation of the immune response in acute kidney injury. Pathogenic bacteria or viruses release pathogen-associated molecular pattern molecules (PAMPs) that bind to pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) on immune cells, stimulate innate immunity. Similarly when cells are injured, molecules that are normally concealed within the cellular compartment are released into the extracellular space. These molecules are referred to as damage-associated molecular patterns (DAMPs), which bind to PRRs to initiate inflammation similar to PAMPs. Immune cells activated by DAMPs and PAMPs are antigen-presenting cells (APCs), including mononuclear phagocytes and neutrophils (PMNs). DAMPs released from cells including chromatin and DNA, uric acid crystals, heat shock proteins, neutrophil alarmins, extracellular matrix proteins, heparin sulfate, laminin, high-mobility group box 1 protein (HMBG1), and others.

and typically mediate pathogen-specific adaptive immunity. In contrast, γδ T cells play an important role in innate immunity at mucosal surfaces and in immunological memory. Upon TCR activation in a particular cytokine milieu, T cells differentiate into natural killer T (NKT) cells, γδ T cells, $CD4^+$ T cell subsets (Th1, Th2, Th9, Th17, Th22, Treg (T regulatory cells), and Tfh (follicular helper T cells)), and $CD8^+$ T cell memory and effector subsets with different extra- and intracellular markers, epigenetic and genetic programs, and metabolic pathways [25, 26].

Under normal physiological conditions, antigen-presenting cells (APCs) and T cells reside in the kidney interstitium [27, 28]. Approximately 30% of the T cells recovered from naïve mouse kidneys are located in blood vessels, as detected by the in vivo immunostaining technique [14]. A substantial proportion of the kidney-resident T cells express a surface phenotype of $CD69^+CD4^+$; CD69 was identified initially as an early lymphocyte activation marker, but it is an important molecule for lymphocyte retention in peripheral organs [29]. High-dimensional mass cytometry revealed that the $CD69^+$ tissue–resident memory T (Trm) cell pool consists of a phenotypically diverse population in the kidney [30]. Naïve T cells have a $CD44^{lo}CD62^{hi}$ phenotype and effector/memory T cells are $CD44^{hi}CD62L^{lo/neg}$. Resident T cells in solid organs (liver, lung, intestine, etc.) have phenotypes distinct from those in lymphoid organs [31–33]. In perfused normal mouse kidneys, resident $CD4^+$ and $CD8^+$ T cells express high levels of CD69 and include a high percentage of effector/memory $CD4^+CD44^{hi}$ $CD62L^{neg}$ T cells, compared to those in lung, spleen, and blood. Compared to splenocytes, kidney $CD4^+$ and $CD8^+$ T cells demonstrate a distinct TCR repertoire (TCR Vβ). There are increased percentages in kidney of $CD4^+$ T cells expressing Vβ2, Vβ7, and Vβ8.1/8.2, double negative αβTCR (lacking CD4 and CD8) T cells expressing Vβ2, Vβ3, Vβ5.5/5.2, Vβ7, Vβ8.3, Vβ8.1/8.2, and Vβ13 chains, and $CD8^+$ T cells expressing Vβ8.3, and Vβ9, when compared with spleen lymphocytes [12].

Evidence of T cells in AKI and allograft kidney rejection

Although T cells contribute importantly to adaptive immunity, they have been detected in acutely injured kidney where innate immunity predominates. Inflammatory cells, including T cells, were detected in biopsies of human cadaveric kidney showing acute tubular necrosis (ATN) 1 week after transplant [31, 32] as well as in kidneys of rodents after IRI [33]. The immunosuppressants mycophenolate mofetil (MMF) and tacrolimus (FK506) were administered to inhibit lymphocyte proliferation, resist transplant rejection, and induce immune tolerance; these agents significantly attenuated reperfusion

injury in the kidney allograft [34, 35]. The association of $CD3^+CD4^+$ T cells and fibrosis in human kidneys up to 1 year after the initial injury suggests that T cells also contribute to acute kidney injury (AKI) to chronic kidney disease (CKD) transition in humans [36, 37]. scRNA-seq showed a highly complex interstitial immune cell compartment during IRI [36]. The interstitial microenvironment early and late in the course of AKI can lead to phenotypic changes in kidney-resident macrophage and T cell populations. T cell subset response following injury may overcome the requirement for TCR-Ag interaction and induces a highly dynamic adaptive response that is associated with injury, regeneration, and fibrosis [36].

T cell subpopulations in mouse kidney IRI

Flow cytometry was used to track resident and/or recruited leukocyte subsets in the injured kidney following IRI with cell counting beads used as an internal microsphere counting standard to obtain absolute cell counts and normalize for differences in cell recovery among samples [12, 13, 38]. Following IRI, kidney $CD4^+$ T cells increased by 45% above sham mice within 30 min, peaked at 1 h, and persisted at 24 h [13]. Rabb and coworkers isolated kidney-resident mononuclear cells from perfused mouse kidneys after IRI (to reduce contributions of intravascular cells) and detected T cell subpopulations by flow cytometry and immunohistochemical stain. Using Percoll® isolation they found between 2.8×10^5 and 1.4×10^6 mononuclear cells in kidneys of male C57BL/6J (5–8 weeks of age) mice. The mononuclear cells included $CD3^+$ T cells (31%), $CD4^+CD69^+$ (30%) T cells, B cells (25%), $CD3^-NK1.1^+$ NK cells (8%), $TCR\alpha\beta^+$ $CD4^-CD8^-$ double negative T cells (23%), $CD4^+CD25^+$ Tregs (1.97%), and $CD4^+CD1d^+$ NKT (10%) cells [12]. $CD4^+$ T cells showed an activated phenotype ($CD4^+CD69^+$ and $CD4^+$LEF-1^+) and were adherent to the endothelial cells, initiating inflammation associated with early IRI [13]. CD69 was expressed at maximum levels at 16–24 h after IRI, followed by a second wave after day 3 corresponding to the repair phase in which TCRβ $CD4^+$ and TCRβ $CD8^+$ T cells may contribute to tissue T cell retention rather than early activation [29].

$CD4^+$ and $CD8^+$ T cells in AKI

$CD3^+$ T cells were found in kidneys predominantly in the peritubular capillaries of the outer medulla as early as 3 h after kidney IRI [39]. These cells contribute to increased vascular permeability and upregulate a host of endothelial-related genes, as detected by the gene array [40]. TNF-α and IFN-γ produced from $CD3^+$ and $CD4^+$ T cells were significantly increased in blood and kidney following IR. $CD3^+$, $CD4^+$, or $CD8^+$ T cell knockout mice and TNF receptor 1α and 1β knockout mice led to an attenuation of the increase in vascular permeability associated with IRI [40]. Adhesion molecules expressed on endothelial cells, P-selectin and E-selectin, contribute to the leukocyte rolling mediating early IR-induced inflammation [41]. Although cytokines and reactive oxygen intermediates in response to kidney IRI are thought to be responsible for Ag-independent activation of T cells, more recent studies have shown that Ag-specific TCRs also participate in the pathogenesis of kidney IRI [42]. Chemokines such as CXCR3 and CXCR5 expressed on $CD4^+$ T cells contribute to the recruitment of inflammatory leukocytes into the kidney following IR and regulate Th1/Th2 immune responses [43]. Short-term mouse antithymocyte globulin (mATG) had a differential effect on T-lymphocyte depletion depending on the lymphocyte compartment. Whereas mATG was effective in T cell depletion in the circulating compartment, it was less effective in depletion of infiltrating T cells from the kidneys resulting in very little difference in injury following IRI [44]. These results suggest the importance of tissue-resident lymphocytes in IRI.

New specific reagents, techniques, and genetic mouse models in T cell biology have established a causal role of T cell subsets in the pathogenesis of AKI. In vivo depletion of $CD4^+$ but not $CD8^+$ cells in WT mice protected kidneys from IRI [45]. The use of genetic mouse models that lack certain types of lymphocytes firmly established the role of T cells in IRI [11, 38]. Using CD4/CD8 knockout mice, there was marked improvement in kidney function with less neutrophil infiltration [46], indicating the importance of $CD4^+$ and $CD8^+$ T cells in kidney IRI. Mice lacking $CD4^+$ and $CD8^+$ T cells (*nu/nu* mice) had an attenuated rise in serum creatinine levels and improved renal histology with less tubular damage after IRI when compared to wild-type controls. Adoptive transfer of $CD4^+$ T cells but not $CD8^+$ T cells into T cell–deficient *nu/nu* mice reconstituted kidney injury after IRI [11], and in vivo depletion of $CD4^+$ cells but not $CD8^+$ cells in WT mice protected kidneys from IRI [45]. $CD4^+$ T cells lacking either CD28 or the ability to produce IFN-γ were inadequate to restore kidney injury [11]. In addition, *Rag-1*$^{-/-}$ mice (lacking B, T, and NKT cells) were also protected from IRI, and adoptive transfer of $CD4^+$ T cells from wild-type mice into *Rag-1*$^{-/-}$ mice reconstituted injury [38]. Transfer of $CD4^+$ T cells from *Ifng*$^{-/-}$ mice into *Rag-1*$^{-/-}$ mice failed to reestablish injury in this model [38]. These results demonstrated a causal role of $CD4^+$ T cells in the early phase of IRI [11, 47] and that IFN-γ produced by these cells is an important $CD4^+$ T cell mediator of IRI [38]. However, some studies have demonstrated that *Rag-1*$^{-/-}$ mice lack kidney protection from IRI [48]. The different published results using *Rag-1*$^{-/-}$ mice are unresolved but may be due to the strain differences or complicated relationship between B and T cells in kidney IRI [49].

Not all studies demonstrated that T cells contribute to IRI. Using GK1.5 antibody to deplete $CD4^+$ T cells or using TCRα-deficient mice (lack functional $CD4^+$ and $CD8^+$ T cells), kidneys were not protected from IR-induced tissue injury or loss of kidney function [50]. It is possible that undetected $CD4^+$ T cells refractory to depletion and/or other T cell subpopulations may contribute to injury and explain these conflicting findings.

T-lymphocytes are direct mediators of cisplatin nephrotoxicity. T cells infiltrated into WT kidneys after cisplatin administration within 1 h, peaked at 12 h, and declined by 24 h. Nu/nu mice had marked improvement in functional and histological injury as well as survival when compared with WT mice that were exposed to cisplatin [49]. However, in a clinically relevant model of cisplatin-induced AKI in mice with cancer, $CD4^{-/-}$ mice were not protected against AKI and had faster tumor growth [51].

Mechanisms of T cells in kidney AKI

Table 6.1 summarizes the role of proinflammatory and antiinflammatory T cell subsets in AKI as well as T cell contributions in the progression of AKI to CKD.

Antigen-independent versus -dependent mechanisms in kidney IRI

The underlying mechanisms of T cells in the pathogenesis of ischemic and nephrotoxic AKI are not well known. Both Ag-independent activation of T cells, through APC-secreting cytokines (TNF-α, IL-6, etc.), chemokines (RANTES, MIP-1), tissue oxygen–free radicals, reactive oxygen intermediates or complement activation [45, 95] and Ag-specific TCR activation participate in the pathogenesis of kidney IRI [39, 42]. TCRαβ-deficient (CD4 and CD8 T-cell–deficient) mice showed significant protection of kidneys from IRI and had improved survival and decreased levels of kidney proinflammatory cytokines [46, 53].

TCR repertoire-dependent and -independent factors contribute to the role of T cells in kidney IRI. When *nu/nu* mice (relatively resistant to IRI) are adoptively transferred with DO11.10 T cells (have a limited TCR repertoire) they have less kidney injury and reduced production of $CD4^+$ IFN-γ following kidney IRI compared with *nu/nu* mice reconstituted with wide type (WT) T cells (have a diverse TCR repertoire). In addition, OVA-specific TCR-transgenic mice—DO11.10 mice immunized with OVA-CFA (complete Freund's adjuvant) had worse kidney function after IRI than control mice treated with CFA alone. These results indicate that (i) a diverse TCR repertoire in the absence of T cell activation was important for kidney IRI in naïve mice, and (ii) the injury observed with restricted TCR repertoire (DO11.10 mice) was no longer attenuated upon antigen-specific T cell activation through TCR in DO11.10 mice [52].

IFN-γ and NGAL in AKI

IFN-γ-producing T cells are critical in the pathogenesis of kidney IRI, and their role in IRI may be linked to neutrophil gelatinase–associated lipocalin (NGAL) [96]. Lee et al. found that NGAL, a 25-kDa protein of the lipocalin superfamily [97] that is covalently bound to gelatinase from neutrophils, is markedly induced in the injured epithelium. NGAL has been recognized as an early AKI biomarker with potentially high sensitivity and specificity [98]. Furthermore, NGAL has been shown to play a role in kidney development [99, 100]. Systemic administration of NGAL protected kidneys from ischemic AKI by inhibition of tubular cell death and induction of antioxidant genes [101–103]. Interestingly, the NGAL encoding lipocalin-2 (Lcn2) gene had the highest fold increase (~ 60) in kidney $CD4^+$ T cells following IRI by unbiased RNA sequencing, and $CD4^+$ T cell–produced IFN-γ was a potential driver for the increased NGAL levels in $CD4^+$ T cells during AKI [96]. Kidneys from NGAL-deficient mice showed worse injury compared with WT mice subjected to IRI, and adoptive transfer of NGAL-deficient $CD4^+$ T cells into CD4-deficient or WT mice led to worse kidney function in kidney IRI. Human kidney $CD4^+$ T cell NGAL also increased significantly in the nonneoplastic kidney parenchyma after ischemic exposure [96]. Taken together, the results demonstrate a novel protective role for $CD4^+$ T cell–derived NGAL in AKI [96].

Th1/Th2 and STAT4/STAT6 pathways in kidney IRI

T cell differentiation is mainly driven by polarizing cytokines that activate JAK/STAT signaling routes, resulting in lineage-specific transcription factor expression and effector cytokine secretion. Th1-type (IFN-γ) and Th2-type (IL-4, IL-5, IL-10, and IL-13) cytokines participate in inflamed tissue injury associated with kidney IRI [104]. IL-12 (through STAT4), IL-4 (through STAT6), TGF-β, IL-6, or IL-21 (through STAT3), and TGF-β and IL-2 (through STAT5)

TABLE 6.1 T cell in acute kidney injury (AKI) and in AKI to chronic kidney disease (CKD) progression.

T cell type	Species	Model and references
Proinflammatory T cell subsets in AKI		
$CD4^+$ and $CD8^+$ T cells	Mouse	Bilateral IRI [11,12,40,46,52]
		Cisplatin [49]
T cells	Mouse	Whole-body IRI [47]
αβ and γδ T cells	Mouse	Bilateral IRI [53, 54]
NKT and $CD4^+$ T cells (Th1/Th2)	Mouse	Bilateral IRI [13, 55–57]
		Unilateral IRI [58]
Type II NKT cells	Mouse	Bilateral IRI [59]
		Unilateral IRI and contralateral nephrectomy [60, 61]
ILCs—NK cells	Mouse	Bilateral IRI [39, 62, 63]
Treg	Mouse	Septic AKI [64, 65]
Antiinflammatory T cell subsets in AKI		
$CD4^+$ T cells, Th1/Th2	Mouse	Bilateral IRI [66, 67]
T and B cells	Mouse	Bilateral IRI [39, 48]
T cells and other leukocytes	Mouse	Bilateral IRI and IPC [68]
Tregs	Mouse	Bilateral IRI [69, 70]
		Bilateral IRI and IPC [72–74]
		Unilateral and bilateral IRI [71]
		Cisplatin-induced AKI [75]
T cells and DN T cells	Mouse	Unilateral and bilateral IRI [44]
DN T cells	Human and mouse	Human nonneoplastic kidney tissue and bilateral IRI in mouse [76]
		Bilateral IRI [77]
		Cisplatin-induced AKI [78]
ILC2	Mouse and humanized mouse	Doxorubicin-induced nephrotoxic injury [79]
		Bilateral IRI [80, 81]
		Unilateral/bilateral IRI [82]
Splenic $CD4^+$ T cells	Mouse	Bilateral IRI with ultrasound [83–86]
γδ T cells	Mouse	Adriamycin nephropathy [87]
T cells in AKI to CKD progression		
$CD3^+$ and $CD4^+$ T cells	Mouse	Unilateral IRI [88]
		AKI to CKD transition model (mild bilateral IRI combined with severe unilateral IRI) [89]
Th1 and Th2 cells	Rat	Unilateral IRI, unilateral nephrectomy, and salt treatment [90]
$CD4^+$/$IL17^+$/$Orai1^+$ cells	Human/rat	Unilateral and bilateral IRI [91]
MAIT cells	Human	Healthy and different kidney diseases' kidney tissue biopsy samples [92]
Tissue-resident NK cells	Mouse	Aristolochic acid nephropathy [93]
γδ T cells	Mouse	UUO [94]
Tregs	Mouse	Unilateral IRI with immediate contralateral kidney nephrectomy (regeneration model) [36]
		Unilateral IRI without contralateral kidney nephrectomy (fibrosis model) [36]

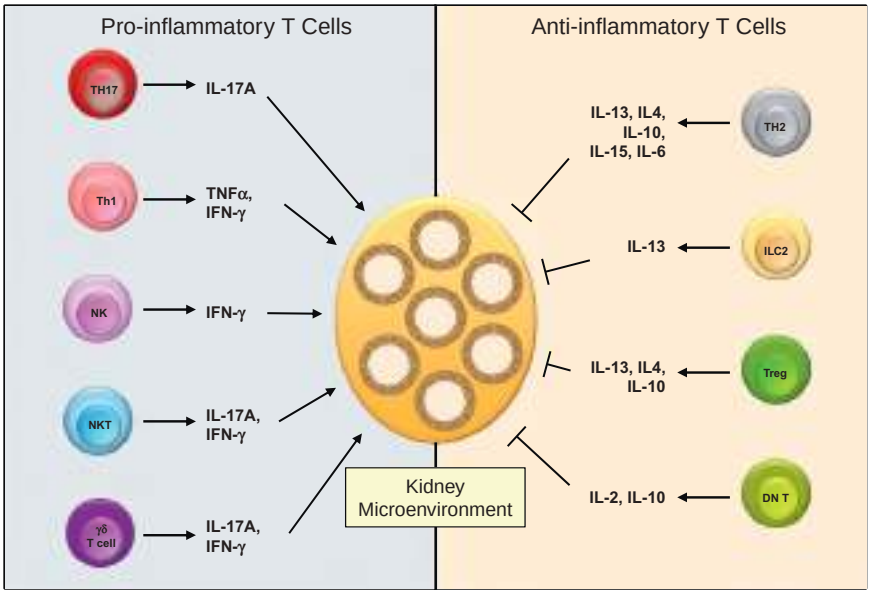

FIG. 6.2 Proinflammatory and antiinflammatory T cells and cytokines in AKI. A number of immune cells are resident to the kidney and upon activation by DAMP-like molecules initiate proinflammatory and antiinflammatory cytokines that influence the extent of injury, repair/regeneration, or fibrosis. *TGF-β*, transforming growth factor beta.

drive the differentiation of Th1, Th2, and Th17 cells and inducible iTregs, respectively [105] (Fig. 6.2). T cells from *Stat4*$^{-/-}$ mice and *Stat6*$^{-/-}$ mice had reduced IFN-γ and IL-4 production response to stimulation [106, 107]. In studies examining the role of CD4^{+} T cell polarization into Th1 and Th2 cytokine-producing cells in kidney IRI, *Stat6*$^{-/-}$ mice had more severe kidney injury with defective Th2 immune response, and *Stat4*$^{-/-}$ mice only had mildly improved function with Th1 defective immune response following kidney reperfusion. *Il-4*$^{-/-}$ mice were similar to *Stat6*$^{-/-}$ mice in their response to kidney IRI. Therefore, the severe injury observed in *Stat6*$^{-/-}$ mice may be due to a deficient Th2 differentiation and decreased IL-4 level [66]. In a separate study, *Il-4*$^{-/-}$ mice had worse kidney function accompanied by higher levels of HO-1, T-bet and later upregulation of MCP-1 [67]. Taken together, Th1/Th2 polarization plays a critical role in the pathogenesis of kidney IRI.

IL-17A and Th17 in AKI

Th17 cells, which secrete the cytokine IL-17, are the prominent lymphocyte population found in rat kidney following IRI [90]. Orai1, a store-operated calcium entry (SOCE) channel, activates Th17 cells, and in rats there was rapid kidney infiltration of Orai1^{+} CD4^{+} T cells following IRI [91]. IL-17 expression was restricted to Orai1^{+} cells. In critically ill patients, Orai1^{+} cells were increased 10-fold and Th17 cells were increased 4-fold in patients with AKI versus those who did not have AKI [91]. Li et al. demonstrated that following kidney IRI, neutrophils produced IL-17A and IFN-γ and the IL-23/IL-17 signaling pathway regulates the IL-12/IFN-γ axis mediating neutrophil migration and tissue injury [108]. Late immunological changes after severe (60 min) kidney IRI include increased CD4^{+} T cell infiltrate and upregulation of inflammatory molecules IL-1 and RANTES accompanied by tubulointerstitial architecture damage 6 weeks after severe kidney IRI [109].

Resident innate-like T cells in kidney IRI: NKT cells and innate lymphoid cells (ILCs, including NK cells, γδ T cells, MAIT cells, and double negative (DN) T cells)

Tissue-resident pool of diverse innate, innate-like, and adaptive lymphocyte subsets contribute to the tissue-resident pool of immune cells and can be activated independently of TCR by cytokines or pattern recognition receptors [14]. The invariant natural killer T (iNKT) cells, *γδ* T cells, mucosal-associated invariant T (MAIT) cells, and double negative αβ TCR T cells display limited TCR repertoires, and they produce innate immune cell–like rapid effector responses by recognizing endogenous or microbe-derived metabolic products (nonpeptide antigen lipids, phosphoantigens, or vitamin B2 metabolites) presented by the nonclassic MHC class I molecules or by MHC Ib molecules [110–112].

Invariant natural killer T (iNKT) and type II NKT cells in kidney IRI

NKT cells are innate-like T cells bridging innate and adaptive immunity by recognizing both exogenous and endogenous lipid antigens presented by CD1d, an MHC class I–like molecule [113]. CD1d-restricted NKT cells include type I NKT cells (TCR Vα14-Jα18-positive, "invariant NKT cells" or "iNKT cells"), which are activated in response to α-galactosyl ceramide (αGalCer), a marine sponge–derived glycolipid. Type II NKT cells (Vα14-Jα18-negative, "diverse NKT cells") are activated predominantly in response to self-glycolipid 3-sulfated β-galactosyl ceramide (sulfatide). In addition, glycosphingolipids, glycosylceramides from gram-negative bacteria and bacterial DNA motifs—CpG oligodeoxynucleotides ds(CpG-ODNs)—are novel antigens that activate NKT cells [114–116]. Mammalian α-linked monoglysylceramides are constitutively produced by immune cells and were found to be endogenous ligands for NKT cells [117]. After encountering antigens, NKT cells rapidly turn into effector cells producing robust amounts of Th1-type (IFN-γ, TNF) and Th2-type (IL-4, IL-13) cytokines/chemokines at the same time, which allow them to amplify and polarize the immune response by modulating immune cell functions [118–123]. Type II NKT cells regulate type I NKT cell–mediated protective autoimmune and antitumor immunity [124] and induce type I NKT cell anergy [125]. Knockout mice used in characterizing the roles of NKT cells include CD1d-deficient mice, which lack both type I and II NKT cells, and Jα18-deficient mice, which lack only type I NKT cells. NKT cells comprise 0.5% of the T cell population in the blood and peripheral lymph nodes, 2.5% in the spleen, and about 10% in the kidney [12, 43, 126].

NKT cells are involved in early tissue damage in AKI [13], but they also have a critical role in inducing tolerance in animal xenograft transplantation models [127, 128]. Systemic administration of αGalCer activates NKT cells and increases endothelial and tubular cell injury in mouse AKI through perforin and TNF-α/FasL pathways independently [55]. Microbial stimuli influence the phenotype and cytokine secretion of NKT cells and also modulate the outcome of kidney IRI [129]. Compared to normal mice, germ-free (GF) mice have more kidney NKT cells and lower IL-4 levels. Furthermore, more $CD8^+$ T cells trafficked into postischemic GF mouse kidneys, which had more severe injury compared to control mice. When bacteria were added to the diet of GF, the extent of kidney IRI was similar to control mice, displaying comparable numbers and phenotypes of T cells and NKT cells [129].

Mouse kidney CD1d-restricted NKT cells ($TCR\beta^+$ CD1d $tetramer^+$) were identified by using CD1d tetramer loaded with PBS57 (an analog of αGalCer with high affinity and specificity for Vα14) provided by the NIH tetramer core facility. CD1d-restricted NKT cells represented 5%–7% of the $CD45^+$ cell population in sham mouse kidneys, and the number significantly increased 3 h after IRI [13]. Disrupting NKT cell function by blockade of NKT-CD1d interaction (αCD1d mAb), depletion of NKT cells (αNK1.1 mAb, PK136), or use of *Jα18*$^{-/-}$ and *CD1d*$^{-/-}$ mice attenuated IRI-mediated neutrophil accumulation and tissue injury [13]. IL-17A induces NKT cell IFN-γ production and promotes $Gr\text{-}1^+$ $IFN\text{-}\gamma^+$ neutrophil infiltration, which promoted tissue injury in kidney IRI [13, 108]. These results indicate that iNKT cells are the major early-acting $CD4^+$ cell subset in the pathogenesis of kidney IRI [13].

Although iNKT cells are an early $CD4^+$ subset that initiates IRI, numerous regulatory factors restrict NKT cell function. The hypoxia/hypoxia-inducible factor2α (HIF-2α)/adenosine A_{2A} receptor ($A_{2A}R$) axis attenuated oxidative stress–induced NKT cell activation and protected kidneys from IRI [130]. Activation of $A_{2A}Rs$ on iNKT cells inhibited the activation of NKT cells and protected liver from tissue injury caused by IFN-γ production and downstream inflammation in liver IRI [131]. Activation of $A_{2A}Rs$ on dendritic cells (DCs) can interrupt the DC-NKT axis in kidney IRI by suppressing NKT cell IFN-γ production through regulation of DC costimulatory molecule expression [132]. Endogenous IL-33 is required for the amplification of the neutrophil-mediated inflammatory response via iNKT cells in the early phase of IRI (1–6 h) [56]. Increased IL-33 interacting with its receptor ST2 on the surface of iNKT cells, together with a key cofactor IL-12, mediated iNKT cell–dependent neutrophil recruitment. IL-33–deficient ($IL\text{-}33^{Gt/Gt}$) mice had reduced early tubular cell injury and subsequent IFN-γ/IL-17A–producing neutrophil infiltrate, with decreased recruitment of NKT cells [58].

Type II NKT cells activated by sulfatide protected kidneys from IRI via hypoxia-inducible factor (HIF)-1α and IL-10 pathways in vitro [59]. The severity of IRI was enhanced mice deficient in type I NKT cells (*Jα18*$^{-/-}$) or type I/II NKT cells (*CD1d*$^{-/-}$), especially in type II NKT cells [59]. Rapamycin recruited NKT cells from spleen to the inflamed kidney through the chemoattractants, CXCR3/CXCL9 and CXCL10, to ameliorate early kidney IRI [133]. The absence of sphingosine 1-phosphate receptor 3 (S1P3) on DCs before (7 days) and after (3 h) IRI prevented DC maturation and promoted a Th2/IL-4 bias resulting in kidney IRI protection through modulation of NKT cell function [57]. Thus, regulation of NKT cell function has become a potential strategy in amelioration of AKI.

Double negative (DN) αβ TCR T cells in AKI and kidney repair

Most human and murine T cells express TCRαβ, but some are double negative for CD4 and CD8 ($CD4^-CD8^-$). These are referred to as double negative (DN) αβTCR T cells ($TCR\alpha\beta^+CD3^+CD4^-CD8^-$). They comprise a very small

(1%–5%) but heterogeneous subset that contributes to normal immune homeostasis, but when expanded they may induce inflammatory tissue damage and autoimmunity [134]. They exhibit effector phenotypes with antiinflammatory (IL-10) and T cell lineage–defining inflammatory cytokine (IFN-γ and IL-17A) and chemokine production. They also display phenotypes of "exhausted" terminal differentiation status with reduced surface CD28, decreased IL-2 production, low levels of T cell receptor excision circles (TRECs), and reduced PD-1 and Helio expression leading to peripheral tolerance. Subsets of DN T cells also display regulatory activity through cell contact–dependent and antigen-specific suppressive effects by inhibiting immune cell populations [134–136].

DN T cells have been studied in the steady state and in response to kidney IRI [12, 76, 137]. Normal mouse kidney DN T cells ($TCR\alpha\beta^+CD3^+CD4^-CD8^-CD1d$ tetramer$^-$) comprise a high proportion (~ 25%) of $TCR\alpha\beta^+$ T cells (similar fraction of $CD8^+$ T cells), more than those in the lymph nodes and peripheral blood [76]. Unlike conventional $CD4^+$ and $CD8^+$ T cells, kidney DN T cells demonstrate an active phenotype with significant expression of $CD62L^{low}$, $CD44^{high}$, and $CD69^{high}$ and important costimulatory molecules CD28 and CD40L, which could give them the potential to interact with local resident APCs. In kidney homeostasis, kidney DN T cells constitutively proliferate at a higher rate, uniquely express IL-10 and IL-27, and suppress $CD4^+$ T cell proliferation in vitro. Kidney DN T cells are early and major responders to IRI in an innate-like pattern; rapid and significant expansion occurred as early as 3 h that was maintained for 24 h after IRI and returned to steady-state level by 72 h following reperfusion [76]. Their rapid response to IRI suggested a role in the lymphoid stress-surveillance program in the kidney [138]. IL-10 markedly increased 3 h after IRI, which contributed to an antiinflammatory effect. Adoptive transfer of DN T cells from FasL mutated generalized lymphoproliferative disease (gld) mice (which have increased DN T cells) protected kidneys from IRI, and the ability of DN T cells to improve IR kidney injury was IL-10–dependent [76]. DN T cells are present in "normal" nonneoplastic human kidney tissue and comprise 18%–61% of αβ T cells (similar to mouse kidneys), as determined by the flow cytometry analysis [76]. However, another study found that only ~ 0.7% of kidney T cells are DN T cells [139]. In patients with lupus nephritis, DN T cells represent a large component of the kidney-infiltrating T cells and are a major source of IL-17 [140]. Further studies are needed to investigate the role of DN T cells in normal human kidneys and in kidney diseases.

β_2-Microglobulin (β_2m) is essential for the expression of both MHC class 1a (H2−K and H2≥D) and most nonclassic MHC class 1b molecules [141]. Kidney DN T cell homeostasis is nonclassic β2m-dependent, and DN T cells utilize MHC-independent mechanisms to respond to external IRI stimuli [77]. The kidney DN T cell number and their activation state in KD-deficient mice (which lack classic H2≥K and H2≥D) and MHC class II–deficient mice were comparable to those of WT mice, indicating that they are not regulated by MHC 1a. Distinct $PD\text{-}1^+$ and $NK1.1^+$ subsets of DN T cells are found in human and mouse kidney parenchyma, which have also been found in thymus and gut epithelium [142]. Interestingly, β_2m deficiency led to the loss of almost all kidney $NK1.1^+$ subsets, and the remaining $PD\text{-}1^+$ subset had impaired proliferation and increased apoptosis [141]. Kidney $PD1^+$ DN T cells and $PD\text{-}1^+$ $CD4^+$ T cells, but not $NK1.1^+$ DN T cells, significantly increased in response to IRI. Similar changes in the DN T cell population, with increased numbers at 24 h that decreased by 72 h, were observed in the cisplatin-induced AKI mouse model [76, 78]. Cisplatin decreased both $PD\text{-}1^+$ and $NK1.1^+$ subsets of kidney DN T cells with more pronounced effect on the $PD\text{-}1^+$ subset. However, further studies are needed to determine whether PD-1-mediated mechanisms seen in Tregs also regulate kidney DN T cells in homeostasis and trafficking in inflamed kidneys [143]. Adoptive transfer of DN T cells from *gld* mice into cisplatin-treated WT mice significantly attenuated tubular injury and the loss of kidney function, indicating that the DN T cell rapid response plays a protective role in cisplatin-induced AKI [78].

Innate lymphoid cells (ILCs)

ILCs include NK cells (or "killer" ILCs), characterized by strong cytotoxic activity, and the "helper-like" ILC subtypes (ILC1-3s and lymphoid tissue inducer (LTi) cells). They are exclusively tissue-resident at steady state, but they modulate T cell responses via cell contact–dependent and TCR-independent mechanisms in barrier organs. They are equipped with a wide array of receptors to sense and respond to the local tissue environment where they reside. They also show organ-specific subset distribution, phenotype, and functional regulation. Different profiles of transcription factor usage and cytokine production characterize three major subsets of "helper-like" ILCs: $T\text{-}bet^+$ $IFN\text{-}\gamma^+$ ILC1s, $GATA3^+$ $IL\text{-}5^+$ $IL\text{-}13^+$ ILC2s, and $ROR\text{-}\gamma t^+$ $IL\text{-}17A^+$ and/or $IL\text{-}22^+$ ILC3s. The major kidney ILC2 populations in mice and humans are ones that produce IL-13 and IL-5 [144, 145].

NK cells not only have effector functions in recognizing and eliminating virus-infected and tumor cells, but they also importantly hold immunoregulatory properties through interactions with immune cells and kidney tubular epithelial cells (TECs) [62, 146]. Tissue-resident NK (trNK) cells display markers of tissue residency/retention such as CD69, CD49a, and CD103 [60]. NK cells comprise ~ 25% of lymphocytes in the healthy human kidney, with

predominant CD56^{bright} NK cell subset, when compared with peripheral blood. Information on trNK cells in the kidney is limited. Parabiosis studies demonstrated the presence of NK cells of two subsets—a circulating NK (cNK) cell subset (DX5$^+$CD49a$^-$) and a minor (15%–20%) tissue-resident NK cell subset (trNK) (DX5$^-$CD49a$^+$) [60]. Kidney trNK cells express lower levels of Asialo-GM1 (AsGM1), as compared to DX5$^+$CD49a$^-$ cNK cells. Therefore, selective (by αAsGM1 Ab) or total (by αNK1.1 Ab) depletion of NK cells allowed the study of trNK cells in kidney IRI. αNK1.1 Ab effectively depleted both trNK and cNK cells and protected against IRI, whereas αAsGM Ab depleted cNK cells and failed to protect against kidney injury, indicating trNK cells played an important role in modulating local response to IR-induced injury [60]. NK cell recruitment occurred in the early phase of kidney IRI (4 h postreperfusion) [39], and endogenous TLR2 ligands released from ischemic TECs with DAMP signaling induced CCR5 expression, which was critical to promoting NK cell recruitment in kidney IRI [62]. Further study showed TEC-NK cell interactions through CD137L-CD137 were important for mediating CXCR2-related neutrophil infiltration of injured kidneys [63]. NKG2D-bearing NK cells as well as expression of Rae-1, a cell surface receptor recognized by NKG2D on NK cells, increased in IRI kidney [61, 147]. NK cells mediated TEC killing (apoptosis) through NKG2D/Rae-1 interactions in vitro, and NK cells directly killed TECs in a perforin-dependent manner in vivo, contributing substantially to kidney IRI [61]. Kidney trNK cells efficiently produce IFN-γ, which is critical for progressive tubulointerstitial fibrosis and chronic kidney disease (CKD). The presence of trNK cells positively correlated with the progression of tubulointerstitial fibrosis in mouse aristolochic acid nephropathy [93]. A large fraction of IFN-γ–producing NK cells (CD56^{bright}) expressing the tissue-resident marker CD69 were found in human kidney biopsy samples from patients with different kidney diseases. The accumulation of these cells correlated with kidney fibrosis, suggesting a role for NK cells in the progression of human kidney fibrosis [148]. In kidney transplant, alloreactive NK cells were involved in T cell–mediated rejection and associated with chronic antibody-mediated rejection and graft loss by priming the adaptive immune system and promoting the migration of other immune cells [149].

Kidney IL-5$^+$ ILC2s are predominantly colocalized with dendritic cells in the perivascular adventitial cuff surrounding the main arterial vessels under homeostatic conditions [150, 151]. IL-33 released by damaged endothelial and epithelial cells is involved in the pathogenesis and associated tissue repair of kidney diseases by binding to its receptor ST2 expressed on Tregs and ILC2s [152–155]. IL-33 or ST2 levels are elevated in cisplatin-induced kidney injury, unilateral ureteral obstruction (UUO), and transplant [156–158]. IL-33 promoted ILC2 and Treg activation and expansion [159], and protection from kidney IRI [82, 144] and cisplatin- and doxorubicin-induced nephrotoxic injury [79, 80, 144]. Depletion of ILC2s rather than Tregs substantially abolished the protective effect of IL-33 in kidney IRI [80]. The protective effect was reversed by the administration of soluble ST2, a decoy receptor, to neutralize IL-33 activity in cisplatin-induced AKI [156]. However, another study showed that there were no differences between tissue injury, fibrosis, or gene expression levels of injury-associated (*Lcn2*), inflammatory (*Cxcl1, Cxcl2*, and *Tnf*), or extracellular matrix (*Col1a1*, *Fn1*) factors in ILC2-reduced, ILC2-deficient, and ILC2-depleted mice, compared to WT mice [150].

A novel Treg-targeted hybrid IL-2 and IL-33 cytokine (IL233) enhances Treg number and kidney IRI protection [81], and it also significantly enhanced the proportion of ST2-bearing ILC2s in blood and kidneys. Adoptive transfer of IL233-treated ILC2s protected mice from IRI [81]. The data support the role of IL233 in protection from kidney injury and also provide evidence that IL233 has strong therapeutic potential.

Mucosal-associated invariant T (MAIT) cells

Mucosal-associated invariant T (MAIT) cells are innate-like T cells that are characterized by a highly restricted $\alpha\beta$ T cell receptor (TCR) that recognizes small molecule antigens in the context (bacterial riboflavin metabolites) of the nonclassic MHC-related molecule 1 (MR1) [160]. The majority of MR1-restricted MAIT cells express the semiinvariant T cell receptor (TCR) α chain Vα7.2-Jα33 in humans and Vα19-Jα33 in mice. They display a memory phenotype and preprogrammed effector activities, which allow them to exert versatile functions from antibacterial activity to metabolic regulation or tissue repair in a TCR-independent manner [161, 162]. They possess a CD69$^+$CD103$^{+/-}$ tissue–resident phenotype in barrier organs including kidney, produce proinflammatory cytokines rapidly [163], and are skewed toward IL-2, GM-CSF, and IL-17A production upon stimulation after kidney transplant [164]. Human tissue-resident MAIT cells in the kidney may contribute to the fibrotic process of CKD via complex interactions with proximal tubule epithelial cells [92].

T cells in the cholinergic antiinflammatory pathway in AKI

The cholinergic antiinflammatory pathway (CAP), centered around the vagus nerve, is a neuroimmune mechanism that involves CD4$^+$ T cells and controls inflammation through neurotransmitters that target immune cells such as

macrophages, which are a major source of proinflammatory cytokines [83, 165–170]. This immunoregulatory mechanism involves the spleen, splenic $CD4^+$ T cells, and α-7 nicotinic acetylcholine receptors (α7nAChRs) found on splenic macrophages to regulate local as well as systemic inflammation in conditions such as rheumatoid arthritis [171], Crohn's disease [172], and experimental AKI [83, 169]. Once CAP activation is initiated, action potentials from the vagus nerve, which is cholinergic, stimulate the splenic nerve innervating the spleen. Splenic nerve terminals, which are adrenergic, are prompted to release norepinephrine locally in the spleen, which in turn binds to β2-adrenergic receptors found on splenic $CD4^+$ T cells. β2-adrenergic receptor activation of acetylcholine synthesizing T cells ($CD4^+$ $CD44^{high}$ $CD62L^{low}$) causes the release of acetylcholine [173]. Acetylcholine binds to α7nAChRs on splenic macrophages, which attenuates the release of macrophage proinflammatory cytokines including TNF-α. The protective effect of vagus nerve stimulation is lost in *Rag-1*$^{-/-}$ mice but restored following adoptive transfer of $CD4^+$ cells [84]. Similarly, adoptive transfer of $CD4^+$ cells treated with salbutamol, a β_2 agonist, protected naïve WT mice from IRI [85]. Thus, neural control of inflammation through the CAP requires specialized acetylcholine-secreting T cells that serve as a critical step in attenuating inflammation in AKI.

γδ T cells in AKI and kidney fibrosis

Tissue-resident γδ T cells contribute to kidney IRI by interacting and promoting kidney infiltration of αβ T cells in the first 72 h of kidney IRI [54]. Mitochondrial damage–associated molecular patterns (DAMPs) released after tissue injury activate γδ T cells [174, 175], and they orchestrate a first-line response to inflammatory signals with rapid cytokine production, such as IL-17A [176, 177]. γδ T cells produce IL-17 and amplify Th17 cell responses following stimulation with cytokines and in the absence of TCR engagement upon. TCR γδ T cell–deficient mice are protected from kidney from IRI and have reduced mortality [54]. However, γδ T cell depletion with the anti-γδ TCR antibody (UC7–13D5) worsened adriamycin-induced progressive glomerulosclerosis [87]. In a clinical study of 20 patients who sustained brief kidney hypoperfusion and reperfusion injury following abdominal aortic repair, there was a significant correlation between tubular injury and a decline of peripheral γδ T cells, but no other T cell subpopulation, within the first 24 h following surgery [178]. In mouse kidney IRI, circulating γδ T cells decreased and γδ T cells in the kidney increased 6.7-fold without alterations in other T cell subsets [178].

Effector γδ T cells also contribute to the fibrotic process and thus progression to CKD. Significant accumulation of the $V\delta1^+$ γδ T cell subset, which expresses human memory T cell marker CD161 and IL-17A, was present in the fibrotic human kidney interstitium. $CD161^+$ γδ T cells also expressed elevated NK cell–associated markers, indicative of a cytotoxic phenotype [179]. The contribution of IL-17A production from infiltrating γδ T cells and $CD4^+$ T cells to kidney fibrosis in the UUO model was through regulation of chemokine RANTES-mediated inflammatory cell infiltration and myofibroblast activation [94] However, IL-17A produced by $CD4^+$ and γδ T cells did not contribute to cisplatin-induced acute kidney injury [180].

Regulatory T cells in AKI, kidney repair, and regeneration

The TCR repertoire of Tregs is important in determining Treg phenotype diversity and allows Tregs to function and maintain homeostasis in the microenvironment, as well as to regulate tissue repair and regeneration [181]. Deficiencies in Treg development or function result in uncontrolled immune response and tissue damage in autoimmune/inflammatory diseases and transplant rejection. A breakthrough in identifying the transcriptional regulator forkhead box P3 (FoxP3) provided important information on specific gene expression programming and epigenetic signature during Treg development [182–184]. Humans who lack FoxP3 develop an autoimmune disease referred to as immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, and mice with mutant FoxP3 (scurfy mice) develop lethal multiorgan autoimmunity [185].

$FoxP3^+CD4^+$ Tregs are primarily generated in the thymus (tTreg) but may also be generated at peripheral sites (pTreg) or can be induced in vitro in cell culture in the presence of transforming growth factor β (TGF-β) [186]. Both Helios and neuropilin-1 (Nrp1) are markers for tTregs [187, 188]. In contrast to mouse Tregs, CD25 and FoxP3 are not sufficient for human Treg identification. The methylation status of the Treg-specific demethylated region (TSRD) within the FoxP3 gene locus can be used for characterizing human Tregs [189]. TCR specificity and affinity determine Treg distribution and suppressive function via Treg interactions with dendritic cells [181, 190]. The CpG motifs in the conserved noncoding DNA sequence (CNS0-3) elements at the FoxP3 locus encode information that defines the size and stability of the Treg population in response to inflammation [191, 192].

Tissue FoxP3^{+}CD4^{+} Tregs have been identified in nonlymphoid tissues (visceral adipose tissue, skeletal muscle, mucosal interface, etc.). Tissue Tregs have unique phenotypes, different TCR repertoires, and distinct functions [193–195].

Treg immunosuppressive mechanisms

Tregs have heterogeneous functions due to their plasticity and capacity to acquire features specific to the type of immune response they encounter, further shaping their environment-specific suppressive functions and ensuring self-tolerance and tissue homeostasis [181, 196, 197] (see Fig. 6.3). Tregs have a broad spectrum of suppressive mechanisms, which are versatile and complementary, to control immune responses. (1) Tregs secrete inhibitory cytokines (IL-10, TGF-β, and IL-35) that result in a broad nonantigen–specific suppression [198, 199], stimulate the generation of tolerogenic dendritic cells, and induce peripheral Tregs (pTregs) (Th3 and Tr1) [200, 201]. (2) Tregs can induce contact suppression. Tregs can kill B and NK cells in a granzyme B- and perforin-dependent mechanism that results in immunosuppression through cytolysis [202, 203]. Human activated natural Tregs express granzyme A and participate in perforin-dependent cytotoxicity against autologous target immune cells [204]. Tregs disrupt Ca^{2+} supply to Teff cells, thus disabling the NFAT- and NF-kB–mediated early TCR activation, resulting in autotolerance [205]. Tregs constitutively express PD-1/PD-L1, which is crucial to FoxP3 expression and interaction with dendritic cells or Teff cells to induce anergy [206]. (3) Tregs use "metabolic disruption" for immunosuppression. The high affinity of IL-2 receptor α (CD25) expressed on Tregs for its ligand (IL-2) competes with Teff cells to deplete IL-2 in the microenvironment, resulting in cytokine deprivation–mediated apoptosis [207, 208]. Ectoenzymes CD39/ENTPD1 and CD73/ecto-5′-nucleotidase expressed on the Treg cell surface can degrade ATP to adenosine, and adenosine can disrupt Teff cell metabolism/activation by activating adenosine 2A receptors (A_{2A}Rs) leading to anergy [209–212]. High levels of intracellular cAMP from Tregs can be transferred to Teff cells through gap junctions, leading to the inhibition of NFAT and IL-2 transcription and cell apoptosis [213]. (4) Tregs modulate dendritic cell (DC) maturation and function by removing the DC surface MHC II/Ag complex (transendocytosis), which causes Teff cell anergy [214], and by triggering Ag-specific peripheral Treg (pTreg) induction

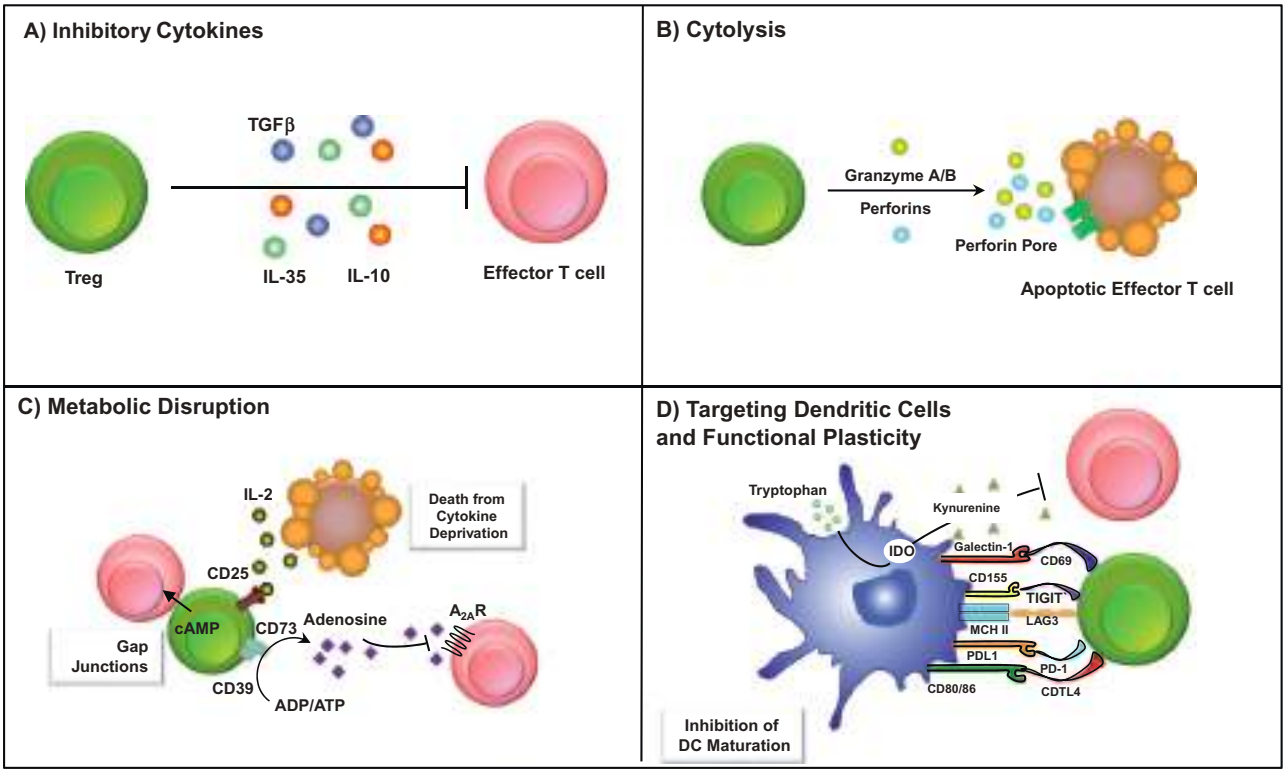

FIG. 6.3 Mechanisms of regulatory T cell suppression of inflammation in AKI. (A) Inhibitory cytokines, (B) cytolysis that includes granzyme and perforin depending on cell killing mechanisms, (C) metabolic mechanisms, and (D) targeting dendritic cells and functional plasticity (see text for description).

through inhibitory costimulatory molecule CTLA-4, lymphocyte activation gene-3 (LAG3), CD223 (band 3 anion transport protein), neuropilin-1 (Nrp1), TIGIT, fibrinogen-like protein 2 (FGL2), and CD69 [215–221]. Tregs condition DCs by initiating indoleamine 2,3-dioxygenase (IDO)–mediated catabolism of tryptophan to kynurenine and through interactions between CTLA4 and CD80/CD86 resulting in the suppression of Teff cells [222, 223]. CD69 binds to its ligand galectin-1 on DCs, which negatively modulates Th17 differentiation and IL-10 secretion through activation of the aryl hydrocarbon receptor (AHR) [29]. Tregs affect secretion of extracellular vesicles and exosomes from DCs promoting tolerogenic immune responses [224]. (5) Tregs have functional plasticity to exert suppressive activity to reduce inflammation. Tregs can regulate Teff cell differentiation through Treg-specific expression of Foxp3, which controls Treg suppressor module molecules (such as CD25, CTLA-4). Tregs can acquire the expression of transcription factors of the Teff population they suppress (Th1, Th2, Th17, or follicular T cells) and travel to the same sites to perform suppression [225, 226]. For example, Tregs upregulate T-bet in type I inflammation [227], IRF4 for the suppression of Th2 Teffs [228], STAT3 to control pathogenic Th17 responses [229], and T cell immunoglobulin with ITIM domain (TIGIT) to control Th1 and Th17 responses [230]. (6) Tregs regulate tissue repair and regeneration in diverse organs [231] by promoting the production of antiinflammatory cytokines/molecules (IL-10, TGF-β, and IDO) and polarizing M1 to M2 macrophages in injured tissue, thereby contributing to healing and regeneration [232, 233]. IL-33R$^+$ Tregs secrete epidermal growth factor (EGF)-like molecule amphiregulin (AREG), which induces mitogenic stimuli and cell differentiation/repair [152]. IL-33 released from injured tissue enhances T cell subset differentiation [234]. Signaling through the IL-33 receptor, ST2, stimulates Tregs and stromal cells, which upregulates reparative AREG production, resulting in inhibiting Ly6C^{hi} monocyte infiltration and reprogramming macrophages to a prorepair phenotype [159, 235, 236]. Tregs secrete keratinocyte growth factor (KGF) to regulate alveolar epithelial proliferation and modulate macrophage response and Th1/Th17 balance during acute injury resolution [236, 237].

Tregs in AKI

The antiinflammatory role of Tregs in AKI has been demonstrated in mouse models including kidney IRI, ischemic preconditioning (IPC), cisplatin-induced nephrotoxicity, and cecal ligation puncture (CLP) septic AKI. The protective effects of Tregs in IRI have been demonstrated by depleting Tregs prior to IRI or by using Treg-deficient mice. PC61 (anti-CD25), a Treg-depleting antibody, was administered 1 day prior to surgery and efficiently depleted CD4$^+$CTLA4$^+$Foxp3$^+$ Tregs in spleen, kidney, and lymph nodes when assessed at 24 and 72 h after kidney IRI. Depletion of Tregs led to a deleterious effect in rat kidney IRI [69]. In a separate study, maximal Treg depletion was achieved when PC61 was administered in naïve mice 5 days before IRI [70]. Kidneys of Treg-depleted mice had worse injury, increased recruitment of inflammatory cells, and increased innate immune-associated cytokines (i.e., IL-6, TNF-α, and TGF-β) after IR, when compared to mice treated with IgG control antibody. Kidneys of Treg-deficient mice (*Rag-1*$^{-/-}$ and Scurfy mice) were more susceptible to IRI and had enhanced inflammatory infiltrate, tissue injury, and loss of kidney function, which was reversed when *Rag-1*$^{-/-}$ mice were reconstituted with WT CD4$^+$CD25$^+$ Tregs, compared with lymph node cells from FoxP3-deficient Scurfy mice 2 weeks before kidney IRI [70].

Reconstitution of *IL-10*$^{-/-}$ Tregs into Treg-deficient *Rag-1*$^{-/-}$ mice demonstrated that Treg protection is IL-10–dependent on kidney IRI. Treg-depleted (PC61) mice had increased kidney tissue levels of IL-2 and IL-10 at 10 days after IRI, which may participate in kidney repair/recovery [70]. Reduced tissue levels of IL-10 were observed in immunosuppressive MMF-treated IRI mice; further studies are needed to identify the mechanism [238].

The expression of ectonucleotidases CD39 and CD73 is important for Treg-generated pericellular adenosine [209]. Adenosine generation and its consequent autocrine effects, mediated by adenosine 2A receptors (A_{2A}Rs) on inflammatory cells, play a critical role in the blockade of the activated T cell proliferation and proinflammatory cytokine secretion in kidney IRI protection [38, 239]. Adoptive transfer of CD25$^+$ Tregs from human CD39-transgenic (hCD39Tg) mice into WT mice conferred greater protection from adriamycin nephropathy (AN) compared with WT CD25$^+$ and hCD39Tg CD25$^-$ T cells [240]. Adoptive transfer of CD73-deficient Tregs (absence of adenosine) or A_{2A}R-deficient Tregs (lack of adenosine responsiveness) failed to protect kidneys from IRI. Activation in vitro of A_{2A}Rs expressed on Tregs via A_{2A}R agonist ATL1222 increased PD-1 levels in Tregs, thereby enhancing the suppressive potential of Tregs to protect kidneys from IRI. However, treating Tregs with a PD-1 blocking antibody before adoptive transfer reversed their protective effects. Taken together, the ability of Tregs to suppress innate immune responses in IRI through a PD-1–dependent mechanism necessitates the generation of and response to adenosine [73]. In addition, PD-1 blocking ligands (PD-L1 and PD-L2) significantly exacerbated kidney IRI, resulting in worse tissue injury and kidney function [74].

Tregs in kidney ischemic preconditioning (IPC)

IPC activates adaptive responses in the initial period of exposure of kidneys to mild ischemia, which renders the kidney resistant to a subsequent sustained episode of ischemia. Adoptive transfer of leukocytes harvested from WT mice 5 days after kidney ischemia into T cell–deficient (*nu/nu*) mice significantly reduced subsequent renal IRI, compared with the transfer of leukocytes from sham control WT mice. The data provided evidence that immune cell phenotype was altered after the initial IRI and lost the ability to cause kidney injury during a second episode of IRI [68]. IPC protects from IRI through increased recruitment of Tregs to the postischemic kidneys [72, 241, 242] and reduced cytokine-secretory responses. Depletion of Tregs (PC61 antibody) before the second, more severe period of ischemia in IPC mice partially or completely reversed the protective effect of IPC, and reconstitution of Tregs in preconditioned Treg-depleted mice restored the protection [72, 242]. It would be interesting in future studies to determine if Tregs isolated from IPC mice are conditioned to prevent IRI compared with Tregs from non-IPC mice.

Tregs in nephrotoxic AKI

Cisplatin is widely used as a chemotherapeutic agent in many cancers, and nephrotoxicity is a common adverse effect. Tregs have a protective role in cisplatin-induced AKI in mice [75]. FoxP3$^+$ Tregs are recruited into the kidney after cisplatin injection. CD4$^+$ CD25$^+$ Tregs attenuate cisplatin-induced nephrotoxicity with broad suppressive effects on many different cell types. Adoptive transfer of Tregs into cisplatin-treated WT and mature T cell–deficient nu/nu mice attenuated kidney injury, reduced macrophage infiltration and proinflammatory cytokines (TNF-α and IL-1β), significantly extended survival time, and reduced kidney dysfunction. Similar to ischemic AKI, PC61-induced Treg depletion prior to cisplatin administration resulted in worse tissue damage and kidney function [75].

Tregs in septic AKI

The cecal ligation puncture (CLP) sepsis-induced AKI model was associated with an increase in IL-10 and Tregs. Interestingly, in contrast to IRI, Treg depletion with PC61 prior to CLP resulted in better survival and kidney function with reduced numbers of apoptotic tubular cells. Distinct pathophysiological mechanisms may account for the differences in the roles of Tregs in kidney IRI and CLP-induced septic AKI. In contrast to kidney IRI, septic AKI was associated with an increase in IL-10 with increased Tregs. Depletion of Tregs prior to CLP resulted in renoprotection [64]. Increases in soluble CD25 and IL-10 in serum of patients with septic AKI indicated the potential association of the paradoxical immunosuppression/paralysis and the development of septic AKI. The data suggested the management of patients with septic AKI should involve maintaining a balance of proinflammatory and antiinflammatory immune responses. Confounding factors and the small number of patients enrolled in this study were limitations of this study [65].

Tregs in recovery/repair from AKI

Significant kidney IRI results in increased incidence of long-term fibrosis. Persistence of T-lymphocyte infiltration, cytokine/chemokine upregulation, and kidney structural damage were identified in moderate or severe kidney IRI. There was a significant increase of CD8$^+$ T cells 2 weeks after IRI; both CD4$^+$ and CD8$^+$ T cells infiltrated the injured kidney 6 weeks after IRI. These T cells had increased expression of markers of activation and effector-memory function (CD69$^+$ and CD44^{hi}CD62L$^-$, respectively) [89]. A similar study demonstrated that adoptively transferred splenic CD3$^+$, CD4$^+$, CD3$^+$CD25$^+$, and CD4$^+$CD44$^+$ cells from IRI mice can traffic to the recipient kidney to cause albuminuria. These data support the concept that lymphocytes can cause long-term kidney progressive dysfunction and serve as potential therapeutic targets for progressive renal diseases [88].

Tregs appear to exert favorable effects in both the early phase and the recovery phase of AKI. Tregs trafficked to the kidneys 3 and 10 days after kidney IRI. Depletion of Tregs (αCD25 antibody) starting one day after ischemic injury increased kidney tissue damage, reduced tubular proliferation, and enhanced kidney T lymphocyte cytokine production at 3 days and CD4$^+$ T cell TNF-α production at 10 days. In addition, Treg infusion had a late beneficial effect on kidney function. Therefore, Treg infiltration following injury during the healing process promotes repair. This is likely mediated through Treg modulation of other T cell subset migration as well as their proinflammatory cytokine secretion [71]. A comprehensive study of gene expression using scRNA-seq in immune cells isolated from kidneys during a 1-year period of progression from kidney injury/regeneration to fibrosis (following unilateral IRI with or without immediate contralateral nephrectomy) provided a detailed profile of the kidney immune cell composition

and the complexity during acute injury, repair, and fibrotic phases. The major early and late injury–induced changes in the kidney included infiltrating (rather than resident) macrophages and T cell populations [4, 243, 244]. T cells showed a highly dynamic adaption overcoming the requirement for TCR-antigen engagement. Kidney-resident Tregs had diverging roles in repair and fibrosis following injury. An early marked increase in tissue-resident IL-33R$^+$ and IL-2Rα$^+$ Tregs protected the kidney from initial damage and late fibrosis development through upregulation of amphiregulin (AREG) and IL-33 receptor ST2 [194, 245]. During fibrosis on day 28, the naïve T cell population was markedly reduced, but Treg genes (*Foxp3*, *IL2ra*, and *Ikzf2*) and tissue-resident Treg genes (*Il1rl1*, *Areg*, *Ctla4*, *Tnfrsf9*, *Tnfrsf4*, and *Tnfrsf18*) had the highest level of upregulation [36]. Tregs displayed divergent genes; proangiogenesis- and tissue repair–associated genes were isolated from regenerating kidneys and proinflammatory- and apoptosis-related genes were isolated from kidneys undergoing fibrosis. The accumulated FoxP3$^+$ Tregs were located in the smooth muscle actin—SMA$^+$ fibrotic area and were close to macrophages in fibrotic mouse and human transplanted kidneys, supporting the findings that Tregs directly influence mononuclear phagocyte phenotype and function to prevent inflammation and promote repair [235, 246, 247]. This study defined the immunological landscape and revealed that resident Tregs exhibited more complexity in the local environment, which warrants further investigation [36]. The valuable information on kidney cellular composition and Treg interaction with macrophages and other cells provides a rationale for therapeutic strategies to promote adaptive kidney recovery and minimize fibrosis progression [36].

Significant mTOR signaling is important in maintaining Treg activity in kidney IRI. mTOR inhibition significantly increased the expansion of kidney Tregs, which was accompanied by increased antiinflammatory cytokine mRNA level in vivo. However, there was no beneficial effect of pretreatment with the mTOR inhibitor rapamycin before IRI on the early recovery of AKI, which may be due to its inhibitory effect on tubular regeneration/repair. However, adoptive transfer of rapamycin-treated Tregs significantly suppressed activating immune cells and myofibroblasts and promoted host Tregs and alternative macrophages, leading to less kidney fibrosis. These findings highlight the therapeutic potential of targeting Tregs in acute and chronic kidney diseases via the mTOR pathway [248].

Treg therapeutic approaches for AKI

The immunoregulatory function of Tregs makes them ideal therapeutic candidates for AKI and kidney transplant. In vitro expansion of autologous Tregs is time consuming (weeks) for collecting sufficient numbers of Tregs and has the added challenge of their specificity, stability, functional activity, and delivery. There are more than 50 active and completed clinical trials testing the safety and efficacy of Treg cell therapy for autoimmune diseases, kidney and liver transplants, and graft versus host disease. The endogenous Ag-specific Tregs are more potent than polyclonal Treg cells, but their expansion in vivo is challenging due to the low number of precursors. Novel technologies were applied to generate Ag-specific Tregs by introducing synthetic receptors to redirect polyclonal Tregs to enhance Ag recognition and to convert Teff to Treg by overexpression of FoxP3 [249]. But the localization and migration of the adoptively transferred Tregs remain a challenge for Treg cellular treatment [250]. IL-2/anti–IL-2 complex, IL-33 agonists, mTOR inhibitors, and DNA methyltransferase inhibitors promote endogenous Treg expansion and represent an alternative option to treat acute organ injury and enhance organ repair [249]. Administration of an IL-2/anti–IL-2 antibody complex robustly increased Treg numbers and attenuated ischemic injury [251]. IL-2 upregulates IL-33 receptor (ST2) expression on Tregs, thus enhancing the effect of IL-33 administration to directly increase Tregs and protect kidneys from IRI [81]. Co-administration of IL-2 and IL-33 expanded Treg numbers in lymphoid organs and protected mice from kidney IRI better than either cytokine alone, and a novel Treg-targeted hybrid cytokine (IL233) provided the best protection [81]. Importantly, administration of IL233 after IRI completely ameliorated kidney injury and mortality and was accompanied by an increase in the ST2$^+$ Tregs in kidneys [81]. Administration of IL233 as late as 2 weeks after doxorubicin-induced AKI increased Tregs and type 2 innate lymphoid cells (ILC2s) in lymphoid and kidney compartments and restored kidney function, further preventing fibrosis by inducing a regenerative program of TECs in a Treg-dependent manner [79]. Other strategies exist to protect kidneys from AKI by mediating Treg number or function, include ischemic preconditioning [72] or pharmacologic treatment with the sphingosine 1-phosphate analog FTY720 [252], phospholipase A2 [253], or the purinergic P2X$_7$ antagonist periodate-oxidized ATP [254].

Tregs in kidney transplant

The important role of Tregs in transplant tolerance has been demonstrated in mice and humans. The enrichment of intragraft Tregs and a greater Treg/CD3$^+$ T cell ratio are associated with favorable graft outcome 2 to 3 years after transplantation [255]. However, other studies reported that the expression of Foxp3, either in blood or in the graft, had no significance in distinguishing between rejection and nonrejection but the demethylation level of the

Treg-specific demethylated region was a favorable predictor of kidney allograft outcomes in a 5-year period post-transplantation [256]. TGF-β produced from allograft kidney tubular epithelial cells induced development of FoxP3$^+$ Tregs and tolerogenic plasmacytoid dendritic cells, promoting immunosuppression or tolerance [255]. Donor-specific Tregs had more suppressive potential in human combined kidney and bone marrow transplant recipients [257]. Type I regulatory (Tr1) cells regulated immunosuppression by secreting IL-10 and blocking MHC class I recognition in allograft kidneys through perforin/granzyme-dependent killing APCs [135]. Engaging the potential immunosuppressive function of Tregs would allow for minimizing the use of immunosuppressive medication in kidney transplant. However, many unsolved problems still remain in applying Treg-based cellular therapeutics for organ transplants, including induction and expansion of a sufficient number of Tregs ex vivo and reducing the risks associated with Treg conversion to effector lymphocytes or an undesirable nonspecific immunosuppressive effect [258, 259].

CD4$^+$ IL-22$^+$ cells in kidney regeneration/repair

IL-22, a member of the IL-10 cytokine family, is produced by Th17 cells, δγ T cells, NKT cells, and innate lymphoid cells (ILCs). Unlike most cytokines, IL-22 primarily targets nonhematopoietic epithelial cells and fibroblasts that maintain barrier integrity and promote tissue regeneration and pathophysiological inflammation in diverse tissues. Cytokines (IL-23, IL-1β, IL-7), aryl hydrocarbon receptor (AhR), and Notch signaling are the important inducers of IL-22 production. IL-22–associated pathology may be due to type I interferon "priming" the tissue and cooperating with other secreted cytokines [260]. Kidney is largely an epithelial organ, and the proximal tubular epithelial cells (PTECs) are the main cells expressing IL-22R and responding to IL-22 through the IL-22R/STAT3 pathway [261, 262]. Serum levels of IL-22 and PTEC IL-22R expression were increased following kidney IRI. IL-22-deficient mice had a slightly increased kidney injury induced by IR, but treatment with IL-22 or overexpression of IL-22 either in IL-22 transgenic mice or by administration of adenovirus expressing IL-22 increased STAT3 phosphorylation in PTECs and reduced tissue inflammation and tubular injury [263]. DAMP signals induced IL-22 production from kidney mononuclear phagocytes via TLR4 upon acute injury stimulation in IRI. IL-22 directly mediated the proregenerative functions of kidney macrophages and dendritic cells in the late phase of IR injury [261]. Increased IL-22 levels were present in remodeling mouse kidneys after UUO. IL-22 had a protective role in chronic kidney injury as IL-22–deficient mice had more disruption of PTEC integrity and epithelial barrier function, but IL-22 did not affect vascular rarefaction or fibrogenesis [264].

Conclusion

T-lymphocytes are a heterogeneous group of immune cells that participate in various phases in acute kidney injury. Recent advances made in our understanding of the identification and functional properties of T-lymphocytes have shed light on the pathophysiology of acute kidney injury, repair, and regeneration. When adaptive mechanisms fail, progressive kidney disease ensues. Specific subsets of lymphocytes participate in different phases of acute kidney injury that in the future may provide opportunities for targeted specific therapies.

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CHAPTER

7

Monocytes and dendritic cells in injury and regeneration

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Monocytes, dendritic cells, and macrophages are often collectively called myeloid cells and belong to the mononuclear phagocyte system [1]. The role of these cells in kidney injury and recovery (regeneration) was poorly understood until the more recent era, as new data concerning myeloid cell biology, lineage relationships, and their association with homeostatic and damaged kidney structures, has come to light. In this chapter, we limit the discussion to information regarding the function of myeloid cells in the context of acute kidney injury (AKI) and recovery. More detailed information regarding their lineage relationships and ontogeny is discussed elsewhere in this book. We begin by defining the prevalent injury models that were used to develop the information regarding kidney myeloid cell function. We also discuss the role of macrophage, dendritic cell (DC), and monocyte phenotypes and the function in the pathogenesis of injury and subsequent recovery.

Models of kidney injury

AKI is an imprecise designation that exemplifies the variety and often unclear causes of renal dysfunction. Previously termed acute renal failure, it is a sudden and significant loss of renal function leading to accumulation of waste products such as blood urea nitrogen and other molecules. The source can be prerenal, intrinsic renal, or postrenal and can result from nephrotoxic agents, sepsis, hypoxia, or other causes. Therefore, a variety of animal models are required to facilitate the study of clinical AKI. Rodent models of AKI are broadly divided into those induced by sepsis, drugs/toxins, ureteral obstruction, and ischemia–reperfusion. In the septic model, a single dose of lipopolysaccharide (LPS) is sufficient to engender sepsis-like symptoms and AKI. However, the extent of damage depends on the dosage. High doses of LPS are fatal, whereas lower doses induce transient kidney dysfunction in a manner that appears to be dependent on inhibition of nitric oxide (NO) and prostaglandins. Inflammatory responses in other models are enhanced when used in conjunction with LPS. Secondly, cecal ligation and puncture can induce sepsis-induced kidney injury, a technique that involves ligation and needle puncture of the cecum. Of note, the correlation of these models with the human syndrome is controversial [2]. Another model used to simulate intrinsic AKI involves the administration of diphtheria toxin (DT) to mice expressing diphtheria toxin receptor (DTR) in proximal tubules. Since rodents are relatively resistant to DT, selective expression of DTR in the proximal tubules generates a targeted insult [3]. The field widely utilizes physical methods of inducing AKI. In the unilateral ureteral obstruction (UUO) model, a single ureter is briefly tied off, either with a silk thread or a nontraumatic clamp, driving tubule dilation, oxidative stress, leukocyte migration, and ultimately fibrosis. Despite obstruction being a less common cause of AKI, the UUO model serves as a valuable tool to study postrenal AKI [4, 5]. Finally, a wide variety of pharmacologic

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agents and toxins are capable of causing AKI in humans and are thus explored in rodent models. Cisplatin, a common anticancer drug, causes dose-dependent nephrotoxicity through direct tubular injury and hemodynamic effects in the kidney [2].

Ischemia–reperfusion injury (IRI) occurs when there is a sudden lack of blood flow followed by reestablishment of perfusion. The loss of blood generates a hypoxic environment that causes cellular damage [6], accounting for approximately 50% of clinical AKI, and is more common in the oldest and sickest patients due to the presence of other comorbidities [2]. In the animal model of IRI, the ischemic insult is induced by clamping the renal pedicle, interrupting the arterial and venous blood flow. While both IRI and nephrotoxic models present with an increase in inflammatory cytokines, the response is more pronounced following IRI. Highlighting phenotypic differences between the models and the challenges facing those developing therapeutics, IL-6 increases insult severity in cisplatin but is protective in IRI [7]. However, cisplatin causes expanded proximal tubule damage as assessed by KIM-1 expression. Finally, IRI enhances the fibrotic response based on collagen, alpha-smooth muscle actin (αSMA), and transforming growth factor-β (TGF-β) production [8]. Therefore, most of the discussion that follows will focus on the role of the innate immune system in kidney injury and regeneration in ischemia–reperfusion models [2].

Injury

Immediately following restriction of blood flow, the resulting hypoxic environment suppresses mitochondrial oxidative phosphorylation leading to a depletion of cellular ATP reserves. The loss of free ATP inhibits sodium and calcium channels, essentially trapping both inside the cell. The increasing calcium concentration overwhelms the cells' ability for sequestration, and chance interactions between calcium and apoptotic receptors initiate apoptosis. As cells die, the damaged tissue begins to release reactive oxygen species (ROS). If restriction ceases, the return of blood flow releases oxygen–free radicals that then participate in lipid peroxidation to cause further damage [6]. Finally, reperfusion allows the influx of inflammatory cells, drawn by the debris and chemoattractants released by the damaged tissue. ICAM-1 and selectin ligands are upregulated postischemia increasing cellular traffic to the distressed area [9]. As the swelling increases, blood flow re-restricts, thus exacerbating the cycle [6].

The innate immune system encompasses monocytic and granulocytic cells of the myeloid lineage bearing pattern recognition receptors (PRRs), as well as complement, defensins, and epithelial surfaces. PRRs are highly conserved and recognize patterns associated with various pathogens. They can be expressed on the cell surface or exist in soluble form in the blood. Toll-like receptors (TLRs) are a significant subclass of PRRs. These receptors recognize many of the commonly known pathogens, such as LPS, peptidoglycan, and bacteria flagella. Common to many myeloid populations, these receptors induce phagocytosis of debris or bacterial invaders. Often the latter are coated in complement, which is composed of inactive soluble proteins that circulate in the blood, activate, and propagate a large-scale inflammatory attack. There are three pathways to induce the complement system, and all converge on the proteolytic C3 enzyme, which can act as a receptor for phagocytosis [10] and catalyzes the subsequent formation of a membrane attack complex. Complement and PRRs have evolved to recognize foreign invaders and facilitate the recruitment and activation of myeloid cells following AKI [11, 12]. Following IRI, tubular damage activates the alternative and mannose-lectin complement pathways, encouraging the recruitment of inflammatory cells to the kidney [13, 14]. Inhibition of proteins involved in complement activation reduces inflammatory cell trafficking leading to better outcomes [13, 14]. Necrotic cells release damage-associated molecular patterns (DAMPs), normally sequestered inside the cell. Cellular biomarkers, such as ATP, heat shock proteins, hypomethylated DNA, and RNA, release into the extracellular space and bind to the PRR, TLR4, resulting in the activation of phagocytosis and polarization toward an inflammatory response [12, 15]. These events result in activation of the NF-κβ and interferon regulatory factor pathways, which are central to inflammation and induction of the adaptive immune system.

Tubular epithelial cells

As a result of the initial hypoxic insult and reperfusion injury, tubular epithelial cells (TECs) are damaged and slough into the intratubular lumen. The remaining damaged TECs upregulate injury markers and secrete damage–associated cytokines [16]. Following the influx of neutrophils, inflammatory monocytes infiltrate within an hour after injury [15]. These monocytes are recruited to the kidney using the CCR2/MCP-1 axis, through P and E selectins and CD11b binding to ICAM-1 [17, 18]. There, cells encounter various DAMPs from necrotic cells and inflammatory cytokines from damaged TECs. DAMPs, such as ATP and uric acid, and hypomethylated DNA signal through TLR, and the NF-κβ pathway, mainly through TLR2 and TLR4, to activate inflammatory macrophages [7, 16, 19]. Inflammatory

cytokines IL-6, TNFα, inducible nitric oxide synthase (iNOS), IL-12, IFNγ, and IL-34 activate the JAK/STAT pathway. Inflammatory monocytes then differentiate into $CD11b^{+}$ $Ly6C^{high}$ proinflammatory macrophages and begin secreting additional inflammatory cytokines and ROS [7, 15]. Additionally, IL-34 and CSF-1 from hypoxic TECs induce macrophage proliferation, generating a positive feedback loop, where injured TECs secrete damage-associated chemokines, recruiting inflammatory macrophages, which increase inflammation and further damage TECs. By 3 days post injury, infiltration by inflammatory monocytes has ceased (Figs. 7.1 and 7.2) [15].

Neutrophils

Responding to the damage signals, TLR2 and nucleotide-binding oligomerization domain-containing proteins 1 and 2 (NOD1/2) present on the cell surface of neutrophils induce migration, and using the adhesion molecules ICAM-1 and P-selectin, these cells move into the kidney, primarily the interstitium, immediately following insult [20–22]. Their presence increases during the first 24 h, but levels remain high past 72 h [22]. Once present, they work to further inflammation and oxidative stress by releasing proteases, cytokines, and reactive oxygen species (ROS) [21]. Neutrophils that detect TNFα but not a pathogen, such as in the case of sterile insult, will degranulate into the extracellular space, further exacerbating the inflammatory environment [12]. They are specialized as first responders to infections and may not be essential for the response to sterile injury [12, 23, 24]. While neutrophils are the initial drivers of inflammation, neutrophil depletion in both cisplatin and IRI does not provide protection, emphasizing the role of other inflammatory cells in injury propagation [23–25]. However, regardless of the stimulus leading to neutrophilic infiltration, they subsequently undergo apoptosis within the kidney and are cleared by macrophages [12].

Dendritic cells

Immature monocytes attracted to the inflammatory signals migrate into the kidney [26]. Once there, evidence suggests that hypoxia activates dendritic cells (DCs), which in turn stimulates NKT cells, CD4 T cells, and cross-present to CD8 T cells [25]. Known as the professional antigen-presenting cells during quiescence, DCs patrol the body in search of antigen. Immature DCs phagocytose antigen and subsequently mature and upregulate T-cell costimulatory molecules [27]. This heterogeneous population is commonly divided into three categories, being conventional DC type I and II and or plasmacytoid DC. While the majority of their response causes increased inflammation, DCs that are activated by the DAMP extracellular ATP mature and develop an antiinflammatory phenotype seen by a decrease in IL-12 production [28]. DCs appear to be a promising therapeutic target. Recent studies

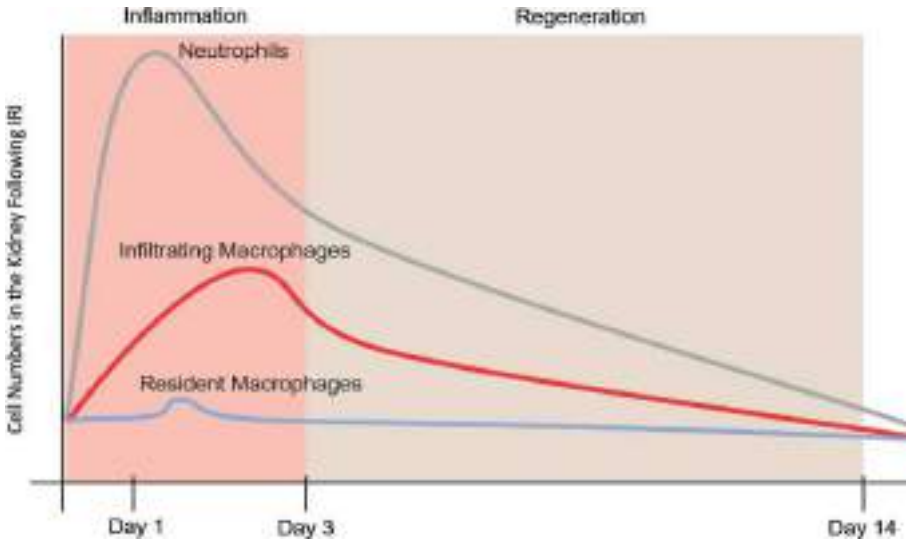

FIG. 7.1 *Influx of myeloid cell populations into the kidney following injury.* Neutrophils quickly enter the kidney following AKI. They are followed by infiltrating macrophages that together propagate inflammation. The resident population does not change significantly. Neutrophils remain high 3 days post injury, but their numbers continue to decrease. Near day 3, the infiltrating macrophages begin to present an antiinflammatory phenotype and the system moves toward regeneration [19].

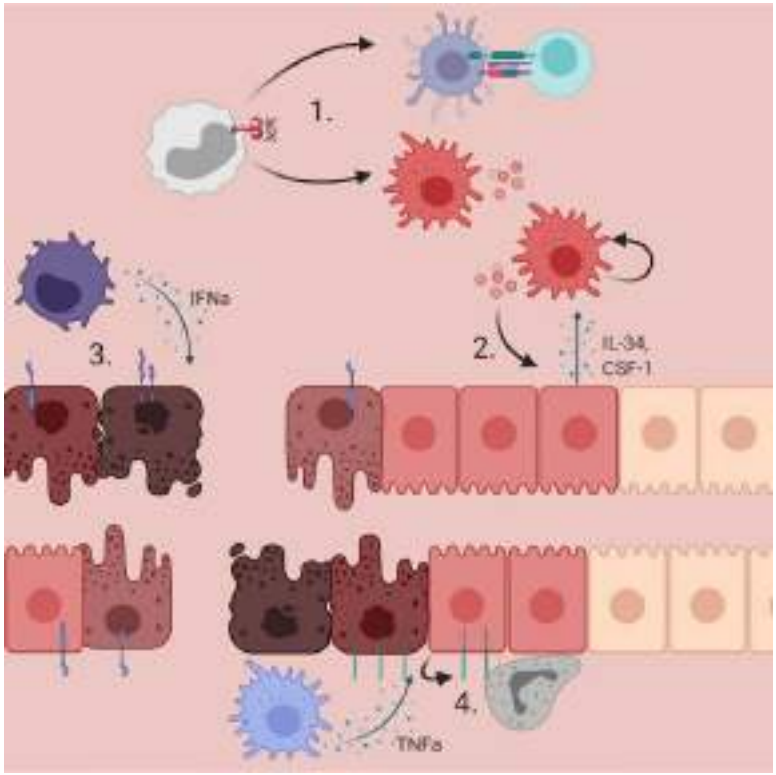

FIG. 7.2 *Roles and interactions of TECs in the propagation of injury and inflammation in IRI.* 1. DAMPs from TECs bind to immature monocytes, resulting in maturation and ultimately T-cell activation and inflammatory cytokine release. 2. Hypoxic TECs release IL-34 and CSF-1, inducing macrophage proliferation and activation. As a result, the macrophages release inflammatory cytokines and induce further TEC damage. 3. Plasmacytoid DCs release IFN-α to induce TEC apoptosis. 4. Resident macrophages release TNFα that causes TECs to upregulate selectins, which infiltrating cells like neutrophils use to move to the injury site. Generated using BioRender.com.

showing ex vivo treatment of bone marrow-derived DCs with a sphingosine-1-phosphate receptor agonist, FTY720, can increase mitochondrial biogenesis. These altered DCs will donate their mitochondria to splenic macrophages and protect against IRI [29].

Typically, in response to viral infections, plasmacytoid DCs are large producers of type I IFNs. In the setting of AKI, both IRI and cisplatin mediated, they are involved in disease pathology, given their massive production of type I IFNs and subsequent inflammation. Specific depletion of plasmacytoid DCs reduces inflammation and immune cell influx. In AKI, the primary function of these cells is the production of IFNα; IRI and cisplatin models and clinical patients present with elevated levels of IFNα [30]. These cytokines cause TEC apoptosis, further activating plasmacytoid DCs and increasing renal inflammation. Activated plasmacytoid DCs can also present antigen to the adaptive immune system, further encouraging inflammation [25]. The depletion of plasmacytoid DCs before injury reduces kidney dysfunction and inflammation [30]. While all three DC subtypes are present during AKI, the focus generally centers on the conventional subtypes, cDC1 and cDC2. Their uncertain and complex ontogeny and potential differentiation from macrophages complicate the role of DCs in AKI.

Macrophages

Since the discovery of an intrarenal myeloid population in 1981, the embryonic origins and classification of these cells have remained controversial because there is considerable overlap of DC and macrophage functions, transcripts, and surface proteins [31]. Initially, their high expression of F4/80 led to their classification as renal macrophages, but co-expression of CD11c led some laboratories to conclude they are DC [32]. However, lineage studies and fate-mapping experiments indicated a much more complex picture, with resident macrophages arising during embryonic life from either the fetal liver or yolk-sac, as observed in other tissues [33–37]. Tissue macrophages are specifically identifiable by surface expression of MertK and CD64 [38]. In addition, the contribution of different lineage precursors may change as a function of age and other variables [35, 39]. Methodological differences in some

studies (e.g., incomplete staining) have also resulted in confusion [35, 37]. For now, the data support the conclusion that the resident population is primarily composed of fetal liver-derived macrophages with some input from the fetal yolk sac early during embryonic life. After birth, there appears to be a contribution from the bone marrow-derived cells over the following first months of life, ultimately reaching an equilibrium in adulthood [40, 41]. While the conversation regarding the ontogeny of the cells continues, this chapter addresses the resident population of CD11b intermediate and F4/80 high cells as macrophages.

This population is composed of ontogenetically different subpopulations. During embryogenesis, $CX3CR1^{+}$ MHC II^{-} macrophages colonize the kidney, forming a contiguous net throughout the entirety of the organ [41]. Beginning near day 10 after birth, the cells in the kidney start to express MHC II, and the resident population is entirely positive by day 28 [34, 37, 39]. Rather than MHC II^{+} cells completely replacing the embryonic residents, embryonic cells persist into adulthood and thus upregulate MHC II expression [34, 37]. Over the following weeks, the embryonic resident macrophages proliferate, and the niche receives supplementation from bone marrow-derived macrophages that enter the kidney and quickly express the same phenotypic and transcriptional markers as their embryonic neighbors, even adopting the same extended lifespan [34].

Based on in vitro experiments, macrophages are traditionally portrayed as either M1 macrophages, activated by TH1 stimuli (LPS or IFNγ) which predominate in the first 48 h after AKI and "exacerbate" injury, or M2 macrophages, polarized by TH2 stimuli (IL-10, IL-4, IL-13) which "resolve" trauma and predominate during the healing phase [42]. This conception of harmful M1 macrophages and helpful M2 macrophages after injury was also based on depletion studies: depleting macrophages before injury or 48 h post injury was protective, while depleting macrophages 48 h post injury exacerbated renal injury [42]. However, the original in vitro categorization of M1 and M2 has not fully translated to an in vivo setting. As macrophages exhibit high levels of functional plasticity, this paradigm of M1 versus M2 macrophages is of limited use in in vivo systems as the behavior of macrophages is now recognized as occurring on a spectrum [15, 39]. Furthermore, the understanding that M2 macrophages are universally protective after a renal injury is too simplistic [15]. For example, in states of persistent injury, M2 macrophages shift from being antiinflammatory to pro-fibrotic, by secreting large amounts of TGF-β and galectin-3, which activate fibroblasts [43]. Although there is interest in M2 cells as a potential therapeutic target after AKI, it is critical to appreciate those different subpopulations of M2 macrophages that can be either beneficial or detrimental after insult. Labeling macrophages as strictly either M1 or M2 macrophages fail to encompass the diverse roles of macrophages within each classification and their phenotypic plasticity based on environmental cues.

The role of macrophages in propagating injury following ischemic insult is complex as demonstrated in various depletion studies resulting in the differing extent of protection or injury depending on the model used and the time of depletion [44, 45]. Facilitated by CCR2 and CX3CR1, monocytes enter the kidney and activation of toll-like receptors and stimulation by IFNγ prompt proinflammatory macrophages at the site of sterile injury, with a possible reliance on the JAK/STAT pathway [18, 46–48]. In response, these cells begin secreting inflammatory cytokines such as IL-1β and IL-12, with the resident population being the largest producers of TNF α [17, 49]. TNFα encourages the TECs to upregulate cell trafficking proteins including selectins and integrins. The continuing increase in inflammatory signals attracts more innate and adaptive immune cells that can be activated by both the dendritic cells and macrophages present within the tissue, extending the inflammatory period. Concurrently, they produce reactive oxygen species via iNOS that act to increase TEC apoptosis and subsequently further increase inflammation while also removing damaged and dying cells [49].

Eosinophils

Finally, the role eosinophils may play in kidney disease is not well understood. Eosinophilia is typically associated with allergic, hematologic, and infectious processes that underlie acute interstitial nephritis. Interestingly, there have been descriptions of eosinophilia in patients with a variety of kidney diseases, including AKI and CKD [50]. Eosinophils are involved in the release of histamine by mast cells, which is known to cause an upregulation of selectins and integrins required for cell trafficking [9, 51]. It is not yet clear whether this is the case during AKI.

Recovery

Although severe injury leads to fibrosis, the kidneys can functionally recover from mild-to-moderate damage, unlike the heart or brain [46]. An estimated 33%–90% of all AKI cases leads to recovery, depending mainly on the underlying cause and severity [52]. The initiation of the inflammation process begins the process of regeneration

and repair, which is governed by a wide array of factors. Generally, the restoration of normal organ function marks recovery. Specific to the kidneys, completed regeneration occurs when the glomerular filtration rate returns to baseline, and the epithelial polarity is reestablished [16]. TECs begin to proliferate within a day of insult and produce cytokines such as CSF-1 and SerpinB2 which skew macrophages toward a pro-reparative, Ly6C^{intermediate}, phenotype, and inhibit migration [9, 16, 53].

Tubular epithelial cells

TECs are the renal cells most sensitive to AKI and likely trigger the immune response immediately following damage [16, 42, 54, 55]. Following the initial hypoxic distress, cellular debris fills the lumen and a failure to clear this debris contributes, at least in part, to delayed recovery [56]. In response, the remaining TECs upregulate expression of MCP-1, GM-CSF-1, IL-4, and IL-34 to induce macrophage trafficking to the kidney and activation toward an antiinflammatory phenotype. During regeneration, antiinflammatory macrophage infiltration increases TEC proliferation through the activation of the TGFβ/Smad7 pathway [57]. Circulating macrophages secrete the protein AIM (apoptosis inhibitor of macrophage), which promotes their survival and additionally binds to intraluminal debris. As macrophages move into the kidney, there is an increase in renal AIM levels. Simultaneously, injured TECs express KIM-1, one of the best-known markers for kidney injury and a receptor for AIM [56, 58]. Binding between AIM and KIM-1 promotes the phagocytosis of debris by TECs. In models of AKI without AIM, the kidneys fail to recover, highlighting the importance of TECs and their expression of KIM-1 [56]. Recovery is complete when the tubule epithelial layer is fully repaired [16] (Fig. 7.3).

Macrophages

The switch by macrophages toward an antiinflammatory phenotype marks the start of the reparative phase. Macrophages begin entering the kidney within an hour of IRI; their numbers are continuing to rise over the

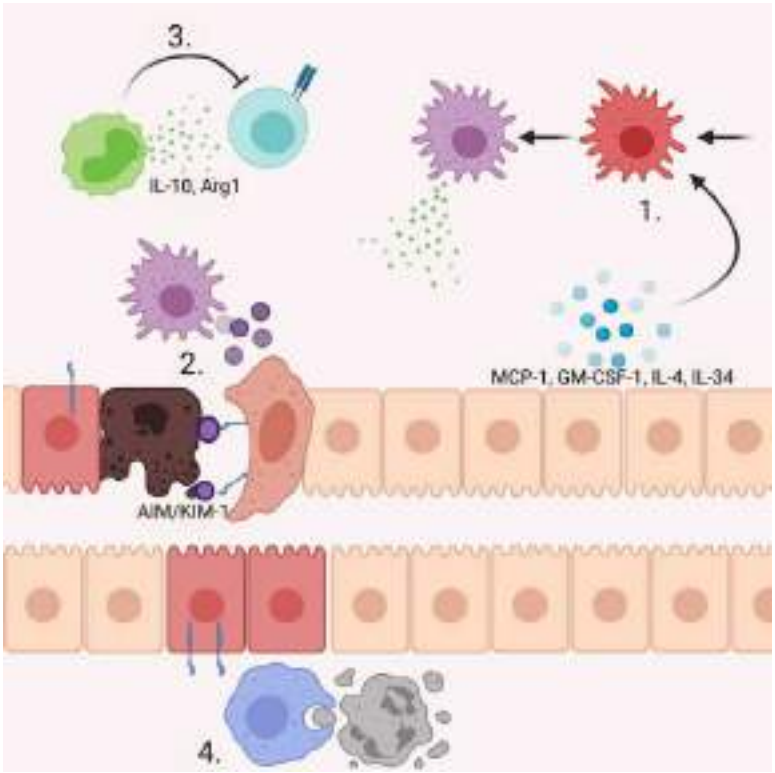

FIG. 7.3 *Roles and interactions of TECs in the amelioration of injury and process of regeneration after IRI.* 1. TECs release MCP-1, GM-CSF-1, IL-4, and IL-34, which encourages macrophage trafficking to the injured site and polarization to an antiinflammatory phenotype. 2. TECs express KIM-1, the receptor for AIM. Binding induces phagocytosis by TECs. Macrophages secrete AIM which binds intraluminal debris. 3. MDSCs suppress T cells and the inflammatory adaptive immune response. 4. Neutrophils initiate apoptosis and are cleared by macrophages. Generated using BioRender.com.

following days (Fig. 7.1) [15]. After acquiring an inflammatory phenotype, these phagocytes work to clear the cellular debris [16]. The interaction between inflammatory cells and necrotic cell debris begins the transition to an antiinflammatory profile in addition to the secretion of the antiinflammatory polarizing cytokine GM-CSF by proliferating TECs [47, 59]. TEC proliferation peaks at 3 days post injury. In the week following injury, macrophages downregulate iNOS, upregulate Arg1, and begin to produce antiinflammatory cytokines IL-10, and TGF-β [43, 60]. Additionally, downregulation of ICAM-1 expression reduces the number of neutrophils and inflammatory monocytes that enter the kidney [15]. Experiments in which antiinflammatory polarized macrophages were adoptively transferred post-IRI caused increased TEC proliferation and decreased inflammation, likely through the secretion of BRP-39 and IL-22, which inhibit TEC apoptosis and induce proliferation, respectively [7, 57]. Antiinflammatory macrophages encourage TEC proliferation, increasing the number of TECs producing antiinflammatory cytokines, thereby generating a cycle similar to before, but in a reparative setting [60]. Infiltrating cells decrease their expression of TNFα and upregulate expression of IL-10 and TGF-β, allowing inflammation to recede in the kidney [59].

Unlike inflammation, which is mainly propagated by infiltrating cells, both infiltrating macrophages and the resident population mediate regeneration. The resident population never takes on an inflammatory phenotype, despite being the largest producers of TNFα after the initial insult [17]. Immediately following injury, resident macrophages downregulate MHC II surface expression, likely limiting T-cell activation. Thereby, these cells recapitulate a developmental phenotype as determined by RNAseq and surface expression [39]. Additionally, they upregulate the expression of the Wnt7b cellular repair pathway [7, 39]. Of the resident population, those that persist from embryonic origins have an enhanced ability to clear immune complexes than macrophages with a bone marrow origin [34]. Additionally, the resident population helps to re-establish sufficient blood flow to the organ by promoting angiogenesis [61]. Studies that deplete the resident population find worse outcomes compared with sham controls [44, 62]. Although understanding of their function remains limited, the resident macrophage population plays an integral role in kidney regeneration.

Myeloid-derived suppressor cells

Similar in function to antiinflammatory macrophages, there has been growing interest in myeloid-derived suppressor cells (MDSCs) both in the kidney and within the immune system as a whole. While most often considered in cancer, they are present in humans after kidney transplantation. This population is composed of activated but immature myeloid cells classified as F4/80 negative, CD11b positive, and either $Ly6C^+$ or $Ly6G^+$, dependent on the subpopulation [61, 63]. This population possesses powerful T-cell suppressing properties, through secretion of IL-10 and Arg1, and the ability to mediate macrophage cytokine expression profiles [63, 64]. Identified in both acute and chronic kidney injury, their manipulation provides a possible future therapeutic target in AKI [63, 64]. Following IRI, treatment with G-CSF increases the number of MDSCs within the kidney and a concurrent decrease in inflammatory cytokine levels, and an increase in antiinflammatory cytokines and ROS [64]. GM-CSF induces granulocyte and macrophage differentiation, whereas G-CSF only induces granulocyte differentiation [65]. This distinction may explain why GM-CSF secreted by TEC leads to increased fibrosis, while treatment with G-CSF, post-IRI, reduced fibrosis [47, 55, 64]. However, clinical studies of human transplants find that $CCR2^+$ MDSCs produce TGFβ1, which, when secreted by macrophages, enhances kidney fibrosis [61, 66]. For this reason and others, the role of MDSCs in kidney regeneration is still uncertain, although it presents a promising therapeutic target.

Failure to recover

In patients, sustained kidney damage or a glomerular filtration rate below 60 mL/min/1.73 m^2 persisting for at least 3 months classifies as chronic kidney disease (CKD) [67]. Concurrently, with the increase in AKI, there has been an increase in the development of CKD. AKI is a significant risk factor for CKD, and the more severe the AKI, the greater the risk. Myeloid cells involved in kidney repair following acute injury promote tissue fibrosis [31], but overall, the process by which AKI progresses to CKD is poorly understood. The current understanding of the mechanisms driving the transition from AKI to CKD includes the development of fibrosis due to myofibroblast activity, endothelial damage, and an influx of immune cells, including myeloid cells causing renal inflammation, tubular epithelial damage, and insufficient repair [68].

Macrophages

Fibrosis involves replacing the damaged epithelial tissue with fibrous connective tissue that cannot perform the same functions as healthy tissue [7, 43]. The avoidance of CKD development following AKI requires either a phenotypic switch or a replacement of pro-fibrotic macrophages and a decrease in numbers to quiescent levels [15]. Otherwise, macrophages will continue to promote extracellular matrix formation and myofibroblast activation or undergo differentiation [15, 69]. Following IRI, three populations of $CD11b^{+}$ macrophages, with differing levels of Ly6C expression associated with either protective or harmful effects after injury, dominate in the kidney at different timepoints. $Ly6C^{high}$ macrophages are the most prevalent 3 h post-IRI and express genes associated with inflammation. While this population is not usually associated with fibrosis given its early appearance and loss of abundance later, it still secretes CCR2 to recruit fibroblasts and MMP-9, a matrix metallopeptidase involved in fibrosis [7, 43, 70]. Subsequently, $Ly6C^{intermediate}$ macrophages are present at 3 h after IRI, predominate at day 3, and express genes associated with wound healing. Finally, $Ly6C^{low}$ antiinflammatory macrophages begin to increase at day 3, peak at day 10, and express genes identified as fibrotic, and they are commonly the pro-fibrotic population [15, 71]. In a chronic injury setting, antiinflammatory macrophages secrete large amounts of cytokines including TGF-beta and galectin-3, which stimulates resident fibroblasts [43, 66, 70]. Immune-deficient mice that receive adoptive transfer of $Ly6C^{-}$ macrophages show increased fibrosis following AKI, specifically by activating fibroblasts [71]. Activated fibroblasts that produce alpha-smooth actin (α-SMA) are called myofibroblasts. These are the prime cell population responsible for the development of the extracellular matrix in the kidney [43]. The precise source of these myofibroblasts in the propagation of fibrosis is controversial. Elegant work from the Humphreys laboratory has suggested that pericytes are a major source for myofibroblasts in models of renal fibrosis [72]. Human biopsy samples from patients with renal disease revealed significant numbers of cells that express both macrophage, CD68, and myofibroblast, α-SMA, markers in fibrotic regions, therefore supporting the notion of a macrophage to myofibroblast transition (MMT) and its subsequent role in fibrosis [69]. Of particular note, the macrophages involved in the MMT appeared to be predominantly antiinflammatory in both human AKI and animal AKI models.

Current therapies

Current treatments for AKI mainly include addressing the underlying cause and supportive therapy. Targeted therapies are undeveloped, but advances in the understanding of immune cells in AKI have generated the potential for novel treatments. Macrophages have risen as a potential target. Global deletion of macrophages or pathways involved with activation, such as CSF-1R or CCR5 [73], have shown promise in mouse models. Additional methods have attempted to alter the balance between pro- and antiinflammatory macrophages. Skewing macrophages toward the antiinflammatory phenotype, either through administering stimuli or adoptive transfer, have been demonstrated to improve renal function after IRI [57]. It is notable that atorvastatin appears to have protective effects in IRI via the downregulation of Th1 cytokines by decreasing M1 density while increasing M2 [74]. A possible consequence of harnessing the M2-like phenotype is the fibrotic response, which may lead to uncontrolled or excess fibrosis. Other groups have identified ways to attenuate fibrosis, which would be essential to preventing damage from cellular therapies. Injection of immunomodulatory cells such as bone marrow-derived stromal cells is capable of attenuating injury by secreting factors that inhibit apoptosis and enhance tubular regeneration [75]. Targeting of neutrophils, the first responders in AKI, has yielded mixed results. One group has found that blocking neutrophil migration reduces injury severity, whereas others fail to see a difference when eliminating neutrophils [7]. Neutrophils still play an integral role in AKI, so their therapeutic potential warrants further investigation. The adaptive immune system may offer additional targets. B cell interactions in AKI are unknown, but studies suggest that their presence may adversely affect outcomes. Two T-cell modulating agents, mycophenolate mofetil and tacrolimus, have been shown to attenuate IRI injury. Mice lacking CD4 and CD8 T cells show moderate protection against affliction [7]. Thus, a careful balance between preventing inflammation postinjury, boosting the antiinflammatory response, and dampening the extent of fibrosis will be paramount in developing treatments for AKI.

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CHAPTER

8

Mast cells in kidney regeneration

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Introduction

Chronic kidney disease (CKD) is a growing health problem worldwide as it leads to the development of renal failure with no treatment options except replacement therapy [1]. CKD can be caused by disorders that affect any of the kidney structures, including glomeruli, renal vessels, and the tubulointerstitial compartment. They are either genetically inherited (e.g., autosomal dominant polycystic kidney disease) or acquired. The latter arise in response to toxic, metabolic (diabetes), hemodynamic (hypertension), post-ischemic, infectious or autoimmune injury. Independent of its origin, the initial injury launches an inflammatory cascade to oppose the injurious insult and initiate tissue repair with eventually limited fibrosis and epithelial regeneration. However, in case of chronic stimulation or defective regulation, the disease will enter into a progressive phase with development of renal fibrosis engendering destruction of individual nephrons and blood vessels. Two-thirds of the nephrons will already have been destroyed before CKD is diagnosed, and from then on the damage progresses to end-stage renal failure regardless of the original cause of injury. Thus, early diagnosis and treatment of CKD risk factors such as diabetes, obesity and hypertension are key public health measures for primary prevention of CKD [2]. In medical care, the relentless decline in renal function can be slowed by therapies based on angiotensin converting enzyme (ACE) inhibitors or Angiotensin-II (Ang II) receptor type-1 antagonists but they are only partially effective [3, 4]. As growing a human kidney by stem cell biology is barely in its first steps [5], to date, replacement therapy either by dialysis or transplantation remains the ultimate therapy when renal failure occurs. Hence, strategies aiming to shift the balance toward repair and kidney regenerative processes are urgently needed in order to develop more effective and specific treatments for progressive renal failure and to halt the ever-increasing number of people requiring dialysis and transplantation. Importantly, researchers have gained a more subtle view of the inflammatory process, realizing that it represents a highly dynamic entity with major contributions to tissue repair and restoration of homeostasis. Therapeutic strategies must therefore be designed to reinforce these positive regulatory actions while suppressing chronic proinflammatory components at the same time.

The presence of an interstitial infiltrate of leukocytes is an essential characteristic of progressive renal diseases. Initially thought as being contributory to disease progression, recent data have made clear that these infiltrating cells also have a variety of positive actions enabling the organism to reduce tissue injury and achieve regeneration. Besides macrophages and T cells, the infiltrate also includes mast cells (MC), suggesting their functional implication [6–8]. MC are of hematopoietic origin. Their precursors, after a short passage in circulation, migrate into tissues where they complete their final maturation under the influence of stem cell factor (SCF) and a variety of other cytokines and growth factors (IL-3, IL-4, IL-9, NGF) [9, 10]. They represent a heterogeneous population of cells that are predominant in tissues facing the external environment (skin, gut…) but can be found in virtually every tissue of the human body including the kidney, generally increasing in number in an inflammatory context [6, 7]. They are now known as highly versatile effector cells with multiple roles in innate and adaptative immunity as well as in the inflammatory processes [11, 12]. Although initially thought to contribute to the pathogenesis of renal diseases, recent

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experimental evidences favor a more subtle view [7, 8, 13]. They indicated that MC can also have beneficial roles including in the reparation and tissue remodeling processes that accompany healing. In this book chapter, we will review the knowledge that has accumulated about the role of MC in renal diseases with a particular emphasis on their positive roles in repair and regeneration.

Basic biology of mast cells

Origin, tissue distribution and heterogeneity of mast cells

MC have originally been described by the German immunologist Paul Ehrlich who noticed in various connective tissues the presence of cells with metachromatic behavior, i.e., the property to change the color of the applied aniline dyes [14]. He named them "Mastzellen" meaning "well-fed" cells due to the presence of numerous cytoplasmic granules, which he thought to contain phagocytosed material or nutrients (Fig. 8.1). Although still many questions remain regarding their differentiation, MC arise from hematopoietic progenitors in the bone marrow [9,10,15]. After a short passage in blood, committed MC progenitors migrate into tissues where they undergo the final stages of their differentiation under the influence of various cytokines and growth factors. The main survival and developmental factor is SCF also known as kit-ligand. This is exemplified by the virtual absence of MC in mice carrying natural mutations in either SCF (WCB6F$_1$-*Kitl*^{Sl}/*Kitl*$^{Sl\text{-}d}$ mice) or its receptor c-kit (WBB6F1- *Kit*^{W}/*Kit*$^{W\text{-}v}$ or C57BL/6-*Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ mice) [16]. Other cytokines and growth factors in the microenvironment may additionally influence the phenotype and number of MC including IL-3, IL-4, IL-9, nerve growth factor (NGF) and transforming growth factor-β1 (TGF-β1), contributing to their striking heterogeneity in various tissues [10,15,17]. While their numbers may be low in certain types of tissues, e.g., in the kidney, they can increase markedly under chronic inflammatory conditions [6].

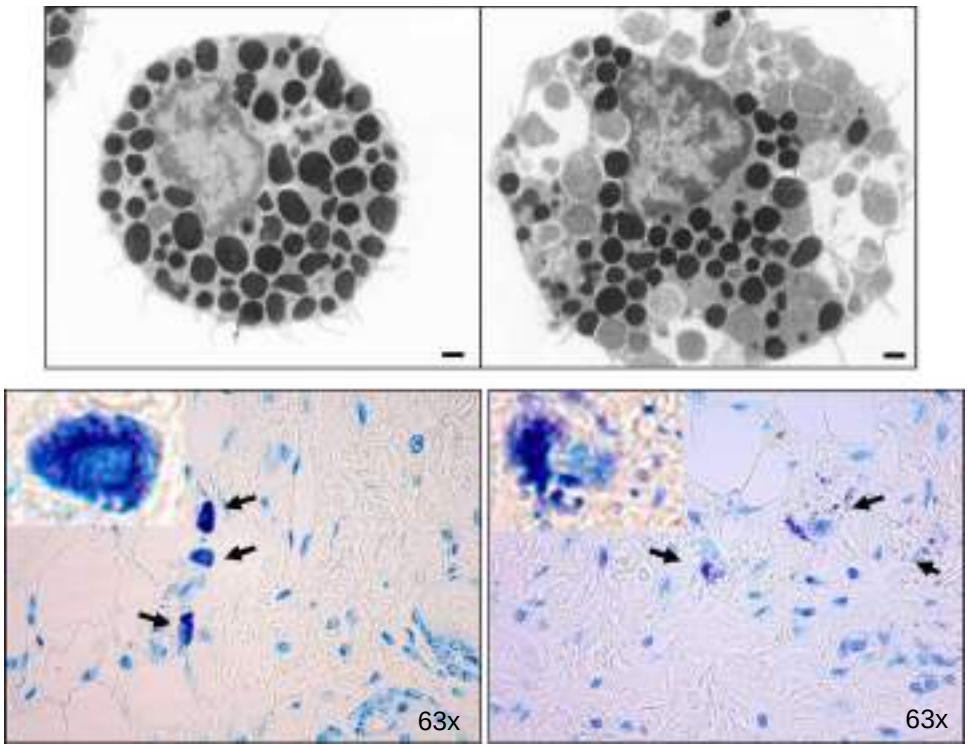

FIG. 8.1 Resting and stimulated MC. The *top panel* show electron micrographs of a resting (*left*) and stimulated (*right*) rat peritoneal MC. Bar = 1 μm. Resting cells contain numerous cytoplasmic secretory granules that are filled with electron dense material. Stimulated cells have undergone partial exocytosis of granular content by a process implying granule–granule and granule–plasma membrane fusion. The *bottom panel* shows granulated and degranulated mouse connective tissue type MC in kidney capsules stained with Toluidine Blue. Note that in degranulated cells, the expelled Toluidine Blue staining granular matrix can be seen in the tissue surrounding the MC. *Inset* shows higher magnification (100X) of one of the MC. *Top panel (left): Courtesy of G. Zabucchi and Maria Rosa Soranzo, University of Trieste, Italy.*

MC are phylogenetically old cells that can be traced back to tunicates [18]. They are round or elongated cells with a diameter ranging between 10 and 20 μm containing a single nucleus and numerous (up to 1000) secretory granules in their cytoplasm storing histamine (also serotonin in rodents), proteoglycans, cytokines and neutral serine proteases (Fig. 8.1). Classically, they are divided into specific subpopulations based on their differential staining properties with various histochemical dyes, which is mainly due to their differential content in proteoglycans [19]. Rodent MC were classified into connective tissue type MC (CTMC) and mucosal MC (MMC), while human MC were classified according to their protease content into tryptase-positive MC (MC_T) and tryptase/chymase-positive MC (MC_{TC}). Even within these defined cell populations, additional phenotypic differences may exist relating, for example, to protease content. Hence, in human kidney a third subpopulation of chymase-positive MC only has been described [6]. New studies support that there is actually a continuum of mast cell phenotypes largely depending on their microenvironment in tissues, resulting also in functional heterogeneity attributed to differential mediator content and receptor expression [10,20,21].

Mast cell mediators

Following activation, MC produce three main classes of mediators (Table 8.1). The first class is contained in secretory granules that are filled with products often ionically bound in the preactivated form to the matrix of proteoglycans such as the negatively charged heparin and chondroitin sulfates. They include vasoactive amines such as histamine and serotonin (the latter in rodents only), a number of neutral proteases (Mast Cell Proteases or MCPT) as well as many lysosomal enzymes such as acid hydrolases [22–24]. In rodents, CTMC express two tryptases (mouse (m)MCPT-6 and mMCPT-7 in mice or rat (r) MCPT-6 and rMCPT-7 in rats), one β-chymase (mMCPT-4 or rMCPT-1) and one α-chymase (mMCPT-5 or rMCPT-5) as well as MC-carboxypeptidase A (CPA3). Additional phenotypic differences depending on the tissue may exist as exemplified by the expression of mMCPT-9 chymase

TABLE 8.1 Mediators secreted by MC[a].

Type of mediator	Mediator
Preformed in cytoplasmic granules	
Biogenic amines	Histamine, serotonin (in rodents)
Proteoglycans	Heparin and/or chondroitin sulfates
Tryptases	α/β tryptases, hTMT (human); mMCPT-6, 7, 11 (mouse); rMCPT-6, 7, 11 (rat)
Chymases	Chymase1 (human); mMCPT1, 2, 4, 5, 9 (mouse); rMCPT-1, 2, 4, 5, 8, 9 (rat); hMC-CPA (human); mMC-CPA (mouse); rMC-CPA (rat)
Lysosomal proteases	Cathepsin D, C, and E
Lysosomal acid hydrolases	β-hexosaminidase, α-glucuronidase
Cytokines	TNF, IL-4, TGF-β1
Matrix metalloproteases	MMP-9
Angiotensinogenase	Renin
Newly synthesized	
Lipid-derived	PGD2, PGE2, LTB4, LTC4, platelet activating factor (PAF)
Cytokines/growth factors	GM-CSF, IFN-α, IFN-β, IFN-γ, IL-1α, IL-1β, IL-2, IL3-6, IL9-10, IL-11, IL12-13, IL14-15, IL16, IL17, IL18, IL22, LTβ, M-CSF, MIF, SCF, TGF-β1, TNF, EGF, VEGF
Chemokines	CCL-1 (TCA-3), CCL-2 (MCP-1), CCL-3 (MIP-1α), CCL-4 (MIP-1β), CCL-5 (RANTES), CCL-7 (MCP-3), CCL8 (MCP-2), CCL-11 (Eotaxin), CCL-13 (MCP-4), CCL-16 (LEC), CCL-17 (TARC), CCL-20 (LARC), CCL-22 (MDC), CXCL-1 (Kc), CXCL2 (MIP-2α), CXCL3 (MIP-2β), CXCL-8 (IL-8), CXCL-10 (IP-10), CXCL-11 (IP-9)
Free radicals	Nitric oxide, superoxide
Blood clotting system	tPA (constitutive), PAI-1 (upon stimulation)

Adapted from references Kalesnikoff J, Galli SJ. New developments in mast cell biology. Nat Immunol 2008;9(11):1215–1223; Mukai K, Tsai M, Saito H, Galli SJ. Mast cells as sources of cytokines, chemokines, and growth factors. Immunol Rev 2018;282(1):121–150; Pejler G, Abrink M, Ringvall M, Wernersson S. Mast cell proteases. Adv Immunol 2007;95:167–255; Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol 2005;6(2):135–142.

in MC of the mouse uterus [25]. MMC contain two β-chymases (mMCPT-1 and mMCPT-2), but do not express tryptases or CPA3. Similarly, rat MC do not express tryptases in MMC, but contain a large variety of chymases (rMCPT-2, 3, 4, 8, 9, 10). Human MC_T express αβ-tryptase, while MC_{TC} express both tryptase and the unique α-chymase present in humans. Following release, the change from acid to neutral pH promotes the dissociation of the various substances from the proteoglycan package albeit at different rates, histamine being released most rapidly. Some highly charged proteins, like chymase, are released more slowly as it sticks tightly to the coreleased proteoglycan matrix, thereby protecting it from natural inhibitors such as α1-antitrypsin, α2-antichymotrypsin or α_2-macroglobulin [26]. Chymase activity partly depends on this association. The proteoglycan content of MC granules also varies in the different MC subtypes. In rodents, CTMC contain heparin, which is not present in MMC. Conversely, MMC express chondroitin sulfates which are not found in CTMC [27]. The dominant proteoglycan in human MC is heparin, which constitutes about 75% of the total, with a mixture of chondroitin sulfates making up the remainder [28]. In humans, the heparin content in MC_T and MC_{TC} is roughly the same. Mice that lack the ability to produce proteoglycans show severe defects in the granule structure of MC, with reduced content of histamine and impaired storage of certain proteases [29].

The second class of mediators are arachidonic acid metabolites that are de novo generated either via the cyclooxygenase or the lipoxygenase pathways [30]. Cyclooxygenase-dependent products include prostaglandins, in particular, PGD2. Lipoxygenase-dependent products include LTB4 and the cysteinyl leukotrienes LTC4 and its conversion products LTD4 and LTE4. All of these lipid compounds have pleiotropic and potent inflammatory actions. For example, PGD2 represents a potent chemotactic agent for leukocytes (eosinophils, basophils) as well as TH2 $CD4^+$ T cell and epithelial cell activation via G protein coupled DP receptors (DP1 and DP2). It promotes arrest of dendritic cell migration (DP1 receptor). It also elicits bronchoconstriction at about 10-fold lower doses than histamine via a still unknown receptor and enhances vascular permeability [30]. The PGD2 in vivo metabolite 15d-PGJ2, binding to the peroxisome proliferator–activated receptor, may also participate in the resolution of inflammation by inducing apoptosis of infiltrating neutrophils and later, of macrophages recruited to clear these apoptotic cells [30,31]. Cysteinyl leukotrienes via binding to G protein-coupled CysLT1 and CysLT2 receptors stimulate prolonged constriction of human bronchi and pulmonary smooth muscle cells. They also enhance vascular permeability, promote bronchial mucus secretion and induce constrictions of arterial, arteriolar and intestinal smooth muscles. LTB4 acts through G protein-coupled leukotriene B4 receptors (BLT1 and BLT2), which may also have some effect on the microvasculature but was reported to be an important inducer of leukocyte (Eosinophil, Neutrophil, MC progenitor) chemotaxis, an action that is shared with cysteinyl leukotrienes [30]. In addition, LTB4 was reported to recruit memory $CD8^+$ and $CD4^+$ T lymphocytes to inflammatory sites [32]. Certain types of MC (CTMC) also produce the lipid-derived platelet-activating factor (PAF) that acts as a potent activator of platelet aggregation and degranulation. It also increases lung resistance and systemic hypotension, suggesting a role for this mediator in systemic anaphylaxis.

The third class of biologically active products secreted by MC includes a large number of cytokines, chemokines and growth factors [22,33]. Some of them are available in a prestored form in secretory granules such as TNF and TGF-β1 for immediate biological action or show some constitutive mRNA transcripts for quick neosynthesis upon stimulation, such as IL-4 [33]. However, the large majority (including the prestored forms) are newly synthesized and released within a few hours of stimulation. Many of them have proinflammatory functions either by directly inducing responses in tissue resident cells or other inflammatory cells through the interaction with cytokine receptors, and/or by attracting other types of inflammatory cells (chemokines) and/or by promoting the proliferation of inflammatory cells within tissues (growth factors). Some of them have potent antiinflammatory activities including, for example IL-10, IL22 and TGF-β1 [33,34].

MC produce also a variety of unclassified products that could be important in inflammatory mechanisms. These include, for example, mediators operative in the fibrinolytic pathway such as tissue plasminogen activator (tPA) produced in resting cells [35], while under certain activating conditions they can also produce PA inhibitor-1 (PAI-1) [36]. Albeit challenged [37], MC have also been reported to represent a unique source of extrarenal renin and thus may contribute to local angiotensin II (Ang II) generation in tissues and related dysfunctions [38,39]. MC also produce nitric oxide and superoxide radicals with known bactericidal, inflammatory and signal-modifying actions [22].

Together, these mediators contribute to the numerous biological activities of MC. Besides the instigation of tissue responses such as vascular permeability and bronchoconstriction initially studied for their importance in allergic processes, these include multiple inflammatory activities such as the recruitment of other inflammatory cells and induction of host defense mechanisms, adaptative (IgE-mediated) immunity and immunoregulatory activities such as Ig isotype switching or T cell polarization as well as tissue remodeling responses. Some of these will be discussed later in more detail, focusing on the mechanisms that may be important in renal inflammation.

Receptors expressed by mast cells

MC are sentinels that express a large variety of cell surface receptors allowing them to respond in different physiological or pathophysiological situations (Fig. 8.2) [40]. Besides the well-studied high affinity IgE receptor known for its implication in allergies or adaptative immune responses [41,42], other immunoreceptors include Fc receptors (FcR) that bind to IgG subclasses, conferring MC responsiveness to stimulation with immune complexes in host defense, but also in autoimmunity [43]. While mouse MC express the activating FcγRIII (CD16), human MC express both activating FcγRIIA (CD32A) and FcγRI (CD64), the latter being induced by low concentrations of IFNγ. Mouse MC also express FcγRIIB (CD32B), while its expression in human MC is not firmly established [44]. FcγRIIB functions as a general inhibitor of cell activation when coaggregated with another activating immune receptor [44,45].

In addition, MC express various receptors involved in innate immunity [40]. These include several types of toll-like receptors activated upon interaction with pathogen-associated molecular products (PAMPs) present in bacteria (i.e., LPS, flagellin), viruses (dsDNA, ssRNA) and parasites. Initial reports focused on TLR2 and TLR4 but many other functional TLR (1, 3, 6, 7 and 9) have been found to be expressed by MC [40]. They also express a variety of receptors for danger-associated molecular products (DAMPs) including the purinergic ATP receptor (P2X7), prostanoid receptors (EP3/4) and TLRs; the latter respond, for example, to HMGB1 (high mobility group box 1 protein), a protein released by injured or necrotic cells. When stimulated through TLR, MC produce cytokines in the absence of degranulation [46]. MC have also been shown to express receptors for various cytokines, chemokines and growth factors [40]. Some of them, such as the chemokine receptor CCR1 or c-Kit, are known to upregulate the responsiveness to other stimuli [33,47]. Others can upregulate the expression of receptors, such as, for example, FcγRI by IFNγ, which can modulate the cellular responsiveness [44]. Receptors for SCF, IL-9 or certain chemokines are important to attract MC to a given anatomical site. Thus, it has been shown that IL-9 transgenic mice are particularly enriched in MC in some tissues including, for example, the kidney [48].

Depending on the type and intensity of the inflammatory stimulus, MC are able to selectively sense and release a specific set of cytokines/chemokines, suggesting that they have the capability of fine-tuning the effector response [40,49,50]. Several forms of infection or tissue injury also lead to the activation of the complement. MC have long been known to express complement receptors such as CR3 (CD11b/CD18), CR4 (CD11c/CD18) or receptors recognizing complement split products such as C3aR et C5aR (CD88) [40]. The expression of these receptors is variable depending on the type of MC (i.e., C3aR and C5aR are expressed only on connective tissue type MC (CTMC, MC_{TC}) and can also be upregulated upon activation.

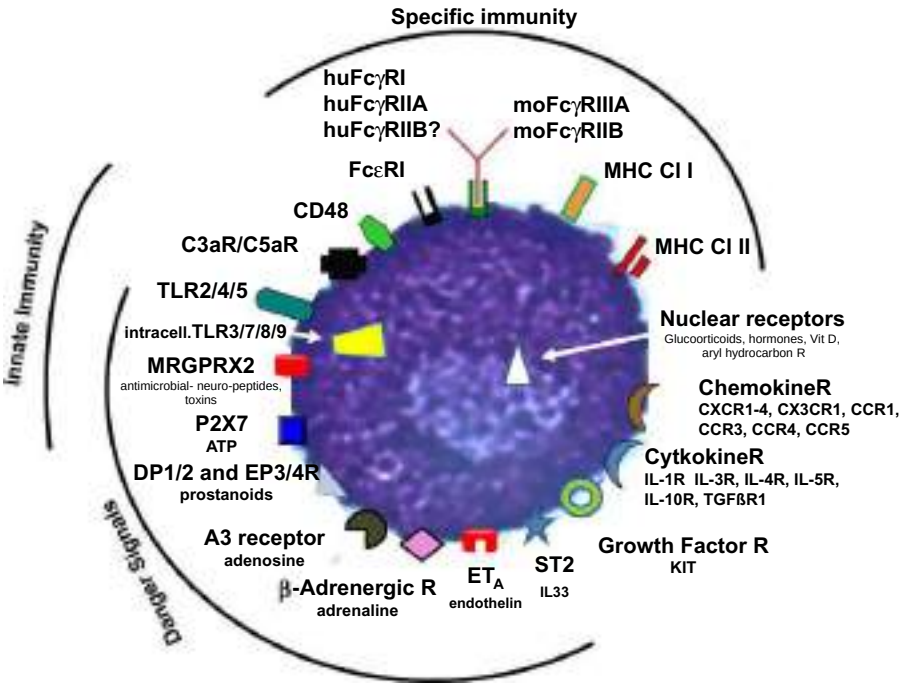

FIG. 8.2 Examples of surface receptors expressed by mast cells. These can be further classified as receptors involved in specific or adaptive immunity as well as danger signals.

A unique receptor called MAS-Related G Protein-Coupled Receptor-X2 (MRGPRX2 in humans and Mrgprb2 in mice) has recently been described to be specifically expressed by MC [51,52]. This G protein-coupled receptor is activated by polybasic compounds such as compound 48/80, wasp-venom-derived peptides such as mastoparan or various neuropeptides (Substance P, Vasointesinal Peptide Neurotensin) and opioids [52,53]. Recent studies validated that MC could represent an important connection between the immune and the peripheral and central nervous system involved in homeostasis and pathogenesis of allergic diseases [54,55].

Model systems to study mast cell functions

A useful model system to study the in vivo function of MC has been the availability of MC-deficient mice. Initially, MC-deficient rodents carrying natural mutations in the c-Kit, the receptor for the MC growth factor SCF, including $Kit^{W}/Kit^{W\text{-}v}$, $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ in mice as well as $Kit^{W\text{-}s}/Kit^{W\text{-}s}$ in rats, have been used to investigate MC biology in vivo [16]. In contrast to $Kit^{W}/Kit^{W\text{-}v}$ mice, the $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice are fertile and not anemic and can more easily be bred including with mice carrying other abnormalities. Although these models are profoundly deficient in MC, due to the defect in c-KIT they also lack melanocytes, interstitial cells of Cajal and show imbalances in a number of other hematopoietic cells such as neutrophils and basophils, meaning that lack of responsiveness in a given experimental model does not necessarily mean that MC play a role. It is therefore necessary to selectively repair the MC deficiency by the adoptive transfer of genetically compatible in vitro–derived bone marrow–derived MC (BMMC) cultured in IL-3 or a combination of IL-3 and SCF. Sometimes they are also generated from embryonic stem or fetal liver cells (ES). Such "MC knock-in" mice can then be used to assess the extent of MC implication or MC mutant in a certain receptor or MC product. While the effective restoration of responsiveness may allow drawing reasonable conclusions about the implication of MC, partial or lack of restoration is more difficult to interpret [16]. Therefore, a series of other c-kit-independent MC-deficient models have been developed recently such as MCPT5-Cre mice crossed with R-DTA or iDTA floxed mice, Hello Kitty Cpa3-Cre; $Mcl\text{-}1^{fl/fl}$ mice, Cre-Master $Cpa3^{Cre/+}$ mice, Mas-TRECK mice or RMB mice [56,57]. These mice are increasingly used to assess the biological roles of MC [56]. Besides models of bona fide MC deficiency a series of other models with genetic deletion of MC-associated products and mediators have proven useful to assess certain MC functions. They include mice deficient in histamine ($HDC^{-/-}$) as well as mice deficient in various granular MC-specific proteases ($MCPT1^{-/-}$, $MCPT4^{-/-}$, $MCPT5^{-/-}$, $MCPT6^{-/-}$, $MCPT7^{-/-}$, $CPA3^{-/-}$ $CPA3^{Y356L/E378A}$ or granule proteoglycans $NDST^{-/-}$ or $SG^{-/-}$ mice [29,56]).

Positive roles of mast cells in inflammation

Besides their well-known role in allergic diseases, MC are implicated in many other chronic inflammatory diseases [49,58]. Yet, they also have many positive roles contributing to immune defense mechanisms against pathogens, toxins, venoms as well as sterile inflammatory responses regulating the number, viability, distribution, phenotype of other immune and tissue cells and repair and tissue remodeling responses [11,49,50,59].

With no doubt the first positive roles of MC put into light relate to their capacity to function in innate and adaptive host defense mechanisms. Because IgE production and MC numbers are markedly upregulated during immune responses to infections with intestinal parasites, this question was one of the first issues investigated in MC-deficient mice. Results clearly showed a more severe disease in the absence of MC in a parasitic nematode infection model with higher larval counts and lower worm expulsion due to deficient mucus production in MC-deficient as compared to WT mice [60]. A series of other helminthic infections, vector-borne parasitic diseases and infection with ixodid ticks have been investigated since then, confirming the important role of MC in the defense against parasites [42,61]. However, the effects of MC in parasitic infections remain controversial as some adverse effects have also been noted and their action also seems to depend on the species of parasite and the infection site [42,61]. Besides parasitic infections, MC play also important roles in bacterial, fungal and viral infections [46,62,63]. First reports on the positive role of MC in bacterial infections appeared in the mid-1990s in two independent studies. Using the model of bacterial sepsis induced by caecum ligation and puncture (CLP) in W/W^{v} mice the authors found that MC-deficient mice were highly susceptible to infection and died within a few days, while > 60% of WT mice survived [64,65]. Further analysis using neutralizing antibodies revealed that MC-produced TNF, rapidly mobilized due to its presence as a preformed mediator in cytoplasmic secretory granules, was a major protective mediator. However, MC-derived TNF

has also been incriminated in the pathogenesis of sepsis and septic shock in a more severe model of CLP using the W^{sh}/W^{sh} strain [66]. In particular, increasing concentrations and especially persistence of high concentrations of TNF during sepsis have been associated with nonsurvival. Hence, many factors including the genetic background, the timing and the intensity of the provoked inflammatory response may influence the disease outcome [49,50]. Precise knowledge of the mechanism regulating the inflammatory response is therefore a crucial issue.

MC are also known to neutralize certain endogenous toxic products such as, for example, endothelin-1, vasointestinal peptide or or exogenous products such as venoms from snakes or bees through the release of MC-derived proteases. This also concerns IgE-mediated responses as, for example, prior immunization and build-up of IgE antibodies protected against exposure to lethal doses of honeybee or Russell's viper [67]. In particular, MC-released proteases, such as MC-carboxypeptidase A, tryptase or chymase, were shown to cleave and neutralize these toxins [67–70]. These results clearly provided evidence for the physiological function of anaphylactic/allergic type of responses, calling for a rethinking of this type of pathology [71].

Numerous studies have indicated the contribution of MC in tissue repair and remodeling responses as well as in fibrosis and scar formation after injury [72–74]. While such tissue repair mechanisms should in principle restore homeostasis, the outcome and the role that MC play in this process is complex, being dependent on many different factors. Yet, it is clear that MC are able to secrete many products impacting tissue remodeling responses [33]. These include growth factors (PDGF, FGF, VEGF…), inflammatory and immunoregulatory cytokines (IL4, IL6, TNF, TGF-β1…), chemotactic factors such as diverse chemokines (CXCL1/2, CCL2, CXCL1…) or small-molecular-weight eicosanoids, which allow to attract and initiate the crosstalk with immunoeffector partner cells. MC-specific proteases such as chymase and tryptase are able to inactivate inhibitors or generate the active forms of metalloproteinases such as MMP-1, MMP-3 and MMP-9 that are important in the degradation of extracellular matrix proteins such as collagen [23,75]. Both chymase and tryptase can generate TGF-β1 from latent forms that are stored in cytoplasmic granules [76,77]. TGF-β1 is paramount in fibrosis, promoting fibroblast activation, myofibroblast differentiation and collagen synthesis [78]. MC tryptase can directly induce these actions on fibroblasts independently of TGF-β1 [79]. An important physiological role of MC-initiated tissue remodeling concerns, for example, their roles in pregnancy, where MC-released proteases mediate the improved implantation and, together with uterine NK cells, spiral artery remodeling necessary for decidualization, placentation and fetal development [80]. MC are also important contributors to the wound healing process in the skin, in particular when wounds are infected with an important role for released IL-6 [81] while their role in sterile wounds remains controversial [82].

MC also participate in hair follicle cycling, a process requiring substantial architectural changes of the skin including proteolysis, angiogenesis and nerve supply rearrangements. Indeed, MC-deficient mice exhibit markedly reduced cycling efficiency [59]. Along the same line, it was found that MC participate in bone remodeling. MC-deficient mutants when subjected to an induced cycle of bone remodeling showed a delayed onset of the remodeling cycle, a decreased duration and extent of the active formation phase as well as diminished new bone matrix synthesis [83]. This probably involves a paracrine mechanism, which influences osteoclast and osteoblast activity. Indeed, histamine was shown to influence the early rapid phase of osteoclast activity during bone remodeling [84].

Mast cells as regulators of immune and inflammatory reactions

Immune cell infiltration is generally considered as being causally linked to disease progression and chronicity. Yet, it has become clear that immune cells can also actively participate in the resolution of inflammation, imposing a highly dynamic view of the inherent processes [85]. Resolution of inflammation involves the production of antiinflammatory cytokines such as IL-10, IL-4, TGFβ, various proteases and lipid mediators such as the resolvins, protectins and maresins produced by regulatory cells such as proresolving macrophages and regulatory T cells among other cell types [85,86]. It also involves the capacity of certain immune cells to shift their phenotype from proinflammatory to antiinflammatory functions. For instance, macrophages shift from proinflammatory M1 to antiinflammatory M2 macrophages Concerning these population the advent of single cell ARN seq analysis has revealed an even greater heterogeneity of these populations in tissues during the various stages of the inflammatory process [87]. MC also participate in the resolution of inflammation. In fact, they can function as cellular sensors or rheostats that readily adapt their responses to the physiological context in the organism [49,50]. They can initiate proinflammatory responses, for example, to fight an infectious process or neutralize noxious products or, on the contrary, initiate responses aimed to limit inflammation engendering repair and prevent further damage. The first evidence for such immunosuppressive functions has come from studies of contact hypersensitivity responses to UVB light in the skin, where it was shown that skin MC mediate UVB light–induced suppression of contact hypersensitivity induced by a hapten, an effect that

was mediated by histamine [88]. Evidence was presented that UV-generated Vitamin D3 activates MC via its nuclear receptor VDR to induce IL-10 production in the absence of degranulation, which curtails proinflammatory mediator production (particularly TNF) and immune cell infiltration [89]. MC have also been known for long to enhance contact hypersensitivity responses (CHS), for example, in a model provoked by the hapten oxazalone, implying an IgE/FcεRI-mediated axis [90]. Other studies, however, indicate that this proinflammatory action can be shifted to an antiinflammatory action under circumstances of high dose oxazolone, which rather promoted an immunosuppressive action of MC, limiting the ensuing T-cell response through IL-2-mediated support to T regulatory cells [91,92]. Similar results have also been obtained in a model of 1-fluoro-2,4-dinitrobenzene–induced (DNFB) induced contact hypersensitivity response in that low doses of DNFB induced a proinflammatory response of MC while high doses of DNFB induced an antiinflammatory phenotype [93,94]. Simultaneous monitoring of MC degranulation and IL10 production in the severe model indicated a strong secretion of IL10. The authors therefore suggest that MC are able to adapt this production depending on the severity of the disease [95]. Suppression of an adaptive T cell response by MC was also shown in a model of experimental malaria induced by bites of Anopheles stephensi. Like for CHS, this was associated with increased IL-10 levels in draining lymph nodes [96]. In a different context, MC, by their ability to interact with regulatory T cells, were also able to promote immunological tolerance to the allograft, an effect that was shown to require IL-9 produced by regulatory T cells [97]. The immunosuppressive functions involved their ability to produce tryptophan hydroxylase-1 (Tph-1), an enzyme which converts tryptophan to serotonin. The ensuing tryptophan exhaustion has a striking antiinflammatory and immunosuppressive function [98]. Likewise, genetic deletion of MC Tph-1 and consequent lack of serotonin also prevented the development of obesity and insulin resistance. Absence of Tph-1-positive MC that strongly infiltrate the adipose tissue and in consequence absence of serotonin slowed down energy expenditure in this tissue [99]. Although MC are largely viewed to represent important contributors to asthmatic inflammation, recent data indicate that some individual MC mediators may also restrain some features of the disease. Hence, mice deficient for the chymase MCPT4 exhibit increased airway inflammation, airway hyperresponsivness and smooth muscle thickening in two models of allergic lung inflammation [100,101]. The protective action was attributed to the ability of MC chymase to degrade the disease-promoting IL-33. Taken together, these examples show that MC, besides their proinflammatory functions, have also immunomodulatory and antiinflammatory roles adapted to a given pathophysiological context [49,50].

Inflammation and kidney diseases: A regenerative perspective

Kidney diseases are initiated by an injury either at the level of the glomerular, the renal vascular or the tubulointerstitial compartment. This initiates a well-orchestrated succession of events that can be caricatured as four phases that may also happen at least in part in parallel: an inflammatory phase, an antiinflammatory phase, a repair phase and a regeneration phase (Fig. 8.3). The initial inflammatory phase predominates in the targeted renal compartment and is characterized by the release of inflammatory products resulting in increased vascular permeability through activation of endothelial cells followed by leukocyte infiltration. Other kidney resident cells including mesangial cells, tubular cells and fibroblasts also become activated. This inflammatory step is followed by an antiinflammatory phase that is tightly associated with the initiation of a repair process and even regeneration in some cases to restore homeostasis. This involves a highly dynamic and complex process that includes, in addition to the infiltrating cells, (i) the proliferation of kidney resident cells (including the putative colonization and differentiation of medullary multipotent stem cells into kidney resident cells), (ii) apoptosis of infiltrating and damaged resident cells, (iii) ECM expansion and tissue remodeling as well as (iv) activation of profibrotic cells such as myofibroblasts for local scar formation, which is eventually limited by antifibrotic processes [102]. Indeed, recent evidence has shown that the kidneys cells have the ability to constantly grow and to regenerate themselves. Instead of a single type of stem cell, this involves more specialized stem cells residing in different kidney segments [103]. Ideally, inflammation will resolve without scarring with restoration of tissue structure and function as has been observed after acute ischemic injury provided the initial injury has not reached the point of no return [104,105]. Passing the threshold leads to persistent cell death and progressing atrophy and is characterized by a temporal association of markers for inflammation and injury [105]. However, the complex interplay between kidney resident and infiltrating inflammatory cells and inflammatory processes remains still incompletely understood [105,106]. Resolution of inflammation requires removal of the initiating stimulus, dissipation of mediators and the cessation of cell infiltration. It is accompanied by the clearance of inflammatory cells by migration and apoptosis and by activation of proteases to restore the ECM in its original form. As the inflammatory episode may have led to the loss of renal parenchyma and extensive fibrosis, adaptative processes may follow through structural hypertrophy and eventually also regenerative mechanisms. In human renal disease, treatment with Ang II or ACE inhibitors can

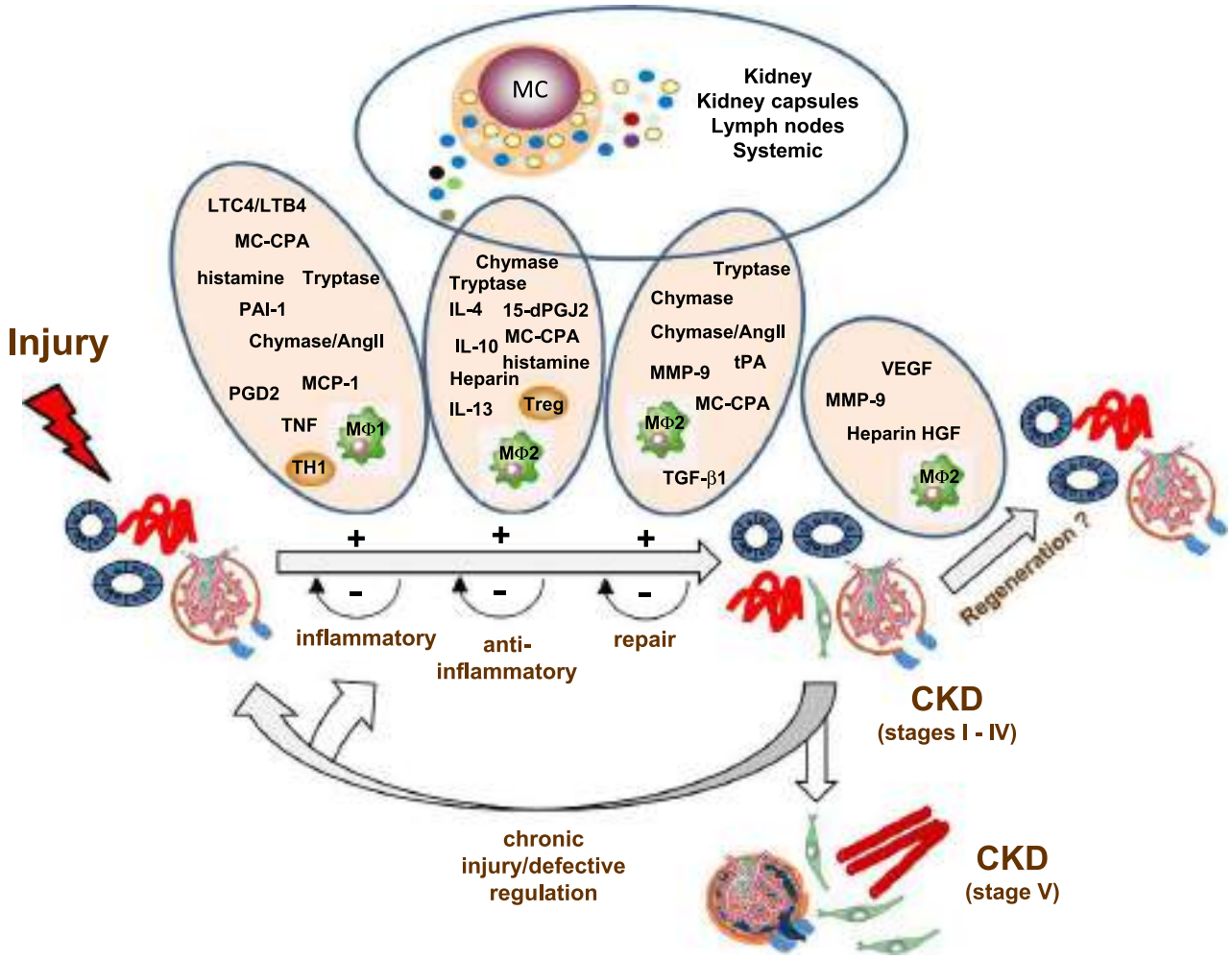

FIG. 8.3 Mast cell actions in kidney diseases. A kidney disease is initiated by an injurious event on kidney tissue structures. This engenders an inflammatory response, which can be classified by caricature into four successive phases: an inflammatory phase, an antiinflammatory phase, a repair phase and a possible regeneration phase. (Note that these phases may take place also in parallel). Together these events are aimed to achieve the healing of local tissue damage. Mast cells infiltrate the kidney tissue especially in the long term but are also present and get activated in the connective tissue surrounding the kidneys. They can also act by releasing inflammatory compounds systemically or by engaging a crosstalk with other immune cells in draining lymph nodes. They act by secreting a wide range of products that participate in the orchestration of these phases, each phase involving a characteristic set of products. Several of these products can have opposite functions during the various phases, indicating a highly complex process that needs to be controlled dynamically involving both positive (+) and negative (−) feedback mechanisms. Any type of chronic injurious event or defective regulation may lead to the installment of an inappropriate inflammatory circuit shifting the balance from repair and regenerative mechanisms to the progression to the final stages of CKD.

slow down fibrosis development. It has become clear from animal studies that an early fibrotic process is reversible in several models of progressive renal disease using ACE inhibition or inhibition of Ang II receptors again underlining the regenerative potential of kidneys [107]. Yet, in case the inflammation does not resolve due to the persistence of the initiating insult or inadequate regulation, iterative cycles of repair take place augmenting the risk of being incomplete, thereby further increasing the risk of persistent injury with nephron and vessel loss and replacement of parenchyma by fibrotic tissue. Although the human kidney can support the destruction of a certain number of nephrons, end-stage renal disease will inevitably develop when more than two-thirds of the renal parenchyma gets lost.

Of note, some actors may be involved during several phases of the process, and some of them may even have a dual role with sometimes apparent opposite aims. If Ang II may exert a main deleterious role with proinflammatory properties during the early inflammatory phase and profibrotic role during the repair phase [107], beneficial actions after interaction with the antiotensin type 2 receptor have also been described [108]. Likewise, MC contribute to the initial inflammation but at the opposite might have beneficial effects in a particular disease context rejoining observations in other inflammatory diseases as eluded to above and as demonstrated by a growing number of experimental evidences (see below).

MC in kidney disease

Localization of mast cells in normal and pathologic kidneys

MC can be detected on the basis of their specific staining properties with metachromatic dyes such as toluidine blue or by immunohistochemical staining with anti-tryptase (recognizing MC_{TC} and MC_T) or anti-chymase (MC_{TC}). These staining methods are based on granular staining and thus have the disadvantage that they do not necessarily recognize activated MC that are degranulated. Indeed, hypervacuolated cells with a paucity of metachromatic cytoplasmic granules have been observed in various inflamed kidneys [6]. Other methods based on anti-c-KIT or anti-FcεRI α chain membrane staining have proven to be inappropriate as other cells are stained including renal tubular cells, interstital cells of Cajal for c-kit [6,109] or macrophages, platelets and eosinophils for anti-FcεRI in humans [11]. More recently, in vivo imaging of MC has also been achieved using RMB mice [57] and by the ability of MC to selectively accumulate fluorescent avidine (avidine-sulforodamine 101) in secretory granules, although this has not been investigated yet in the context of renal diseases [95,110].

Table 8.2 provides some examples from studies in the literature that report the relative increase in MC counts in various renal diseases as compared to normal kidneys [6]. It can be noticed that while MC in normal kidneys are sparse, all cases of progressive kidney diseases are associated with significantly elevated numbers of renal MC. They are localized predominantly in the interstitium where they concentrate in areas close to tubules and blood vessels and are included in mononuclear cell infiltrates associated with areas of fibrosis [6]. Although some periglomerular localizations are also noted, MC have never been found within glomeruli. Furthermore, staining with tryptase and chymase-specific antibodies showed that both MC_{TC} and MC_T are present with a predominance of the latter, although the ratio of the various MC subtypes varied with the renal disease. In rejected kidney transplants, a third MC type stained with anti-chymase only has also been found [111]. In most cases, the increase in MC numbers correlated with the concentrations of serum creatinine and with fibrosis and thus poor outcome. It should be mentioned that MC are also found in significant numbers in the connective tissue of the kidney capsule surrounding the renal parenchyma both in normal and inflamed kidneys.

MC in human renal diseases

MC, by virtue of their mediators and responsiveness to many inflammatory stimuli, could participate in various steps of the inflammatory cascade in kidney diseases either through systemic or local activation. Through mediator secretion, they may participate in the removal of the injurious insult and in the repair processes, or alternatively participate in injurious processes and disease progression. Although MC numbers are low in normal kidneys, in most human nephropathies they are increased correlating with the advanced stages of disease (Table 8.2). In the blood evidence has been presented showing that serum levels of the MC growth factor SCF were elevated in patients with end-stage renal failure [112]. A recent study indicated that serum tryptase is also increased and significantly correlated with the progression to end-stage renal failure in different chronic kidney diseases [113]. Generally, these

TABLE 8.2 Examples of MC counts in various nephropathies[a].

Diagnosis	MC counts
Normal kidney	0.5 ± 0.2 cells/mm^2
Benign nephrosclerosis	1.6 ± 0.4 cells/mm^2
Membranous nephropathy	6.5 ± 7.7 cells/mm^2
Lupus nephritis	12.9 ± 15.8 cells/mm^2
ANCA-related nephropathy	18.5 ± 21.1 cells/mm^2
Membrano-proliferative nephropathy	19.8 ± 14.2 cells/mm^2
IgA nephropathy	21.3 ± 17.7 cells/mm^2
Diabetic nephropathy	33.0 ± 33.8 cells/mm^2
Renal allograft rejection	12.7 ± 1.2 cells/mm^2

[a]*Examples of MC counts were taken from data assembled in Ref. [6].*

correlative data led to the idea that MC had an aggravating role, likely because of their negative connotations in various chronic inflammatory and/or autoimmune diseases. However, the simple correlation of MC numbers, MC-related mediators or growth factors with disease severity is clearly insufficient to judge the role of MC. As indicated above, the described process in kidney diseases appears complex, requiring a finely tuned chain of events composed of proinflammatory components but also antiinflammatory and repair components during the resolution phase. Thus, beneficial actions required during the initial phase may not necessarily play the same role during the late phase and could be counterproductive when they are not downregulated. As an example, one can mention the fibrinolytic system. While during the initial phase of injury, formation of fibrin clots is necessary, these clots need to be resolved during the remodeling/repair phase by the action of fibrinolytic enzymes. In this context, MC have been shown to release many products with fibrinolytic action including tryptase and tPA [36] and MC-mediated fibrolytic activity was found to protect in a murine model of immune complex mediated glomerulonephritis [114]. Another example is the inactivation of hepatocyte growth factor (HGF) by human MC chymase or murine MCPT-4, which during the initial injurious phase may be necessary to limit proliferation of kidney resident tubular cells, while during the later phase this may prevent epithelial cell regeneration [115].

Only a few studies in humans tried to further investigate whether MC in addition to being correlated in numbers with disease severity may be implicated functionally. In a study with renal allograft patients, retrospective clinical data showed that patients with a history of allergy showed more severe episodes of rejection compared with nonallergic patients. This indicated that the inappropriate activation of MC may promote rejection and break tolerance [116]. Few studies have addressed the question whether infiltrated MC are actually activated and by which mechanism. Review of the histological analysis in the literature usually shows perfectly granulated toluidine blue stained or tryptase-positive cells that do not seem to be degranulated. It is therefore possible that MC, although correlated with disease severity, may not play a role in the associated pathological process in the long term and only fulfill a role of sentinels without being involved in the chronic development of the disease. Some studies, however, have reported that MC become activated notably in diabetic nephropathy where MC were reported to be frequently degranulated [117]. These studies further showed that MC strongly expressed type VIII collagen, suggesting that they may directly contribute to extracellular matrix accumulation. Another study showed that with progression of diabetic nephropathy, MC became more and more degranulated and were found to increasingly produce chymase, renin, TGF-β1 and TNF-α [118]. Similarly, in cases of severe IgA nephropathy, immunohistological staining with chymase frequently shows the enzyme in the area around MC, indicating that they have liberated their content at least partially [119]. Furthermore, as stated above, tryptase levels were shown to be elevated in the serum of patients with different nephropathies, indicating also systemic distribution of MC mediators [113].

Another approach to investigate their functional implication was by examining MC subtypes. Two studies reported that the relative increase of MC_{TC} as compared to MC_T was related to the decline in renal allograft function and fibrosis development, suggesting that the pathologic function may be associated with the connective tissue type known to release chymase. The possible relevance of the association with fibrosis is highlighted by data showing that human chymase or its murine functional counterpart mMCPT-4 can function as a major alternate pathway for Ang II generation in addition to ACE [120]. Ang II has been recognized as a major factor involved in renal fibrosis development. Hence, MC-released chymase may play an important alternative to ACE for Ang II generation. Increased chymase expression was indeed noticed in patients with diabetic nephropathy [121] and this was also confirmed in studies in mice where a switch from ACE-dependent Ang II generation to chymase-dependent generation occurred [122] and where inhibition of chymase retarded the development of albuminuria [123]. Recently, a mass spectroscopy comparative analysis between healthy kidneys and kidneys from CKD patients clearly showed dysregulation of RAS in CKD dictated by high chymase-mediated Ang II formation although this could simply reflect the high MC content [124]. In addition, MC may also play a role in ACE-dependent Ang II upregulation, as it has been shown that cardiac MC and the human MC line HMC-1 can release Ang I generating (the substrate of ACE) renin by degranulation [125] although this was somewhat disputed by others [37]. Another profibrotic action may come from the action of tryptase. This protease, in association with heparin, was shown to promote proliferation of human renal fibroblasts as well as the synthesis of fibronectin and type I collagen, likely by its action on PAR-2 receptors [79].

Taken together, data from several human kidney diseases indicate a correlation of the observed MC infiltrate with disease severity. Although some putative mechanisms of the role of MC have been suggested, a clear demonstration of their functional involvement is still missing. Therefore, one approach to obtain additional information comes from animal studies with MC-deficient strains. Results obtained from these studies are summarized in the following. They clearly support also positive actions of these cells in kidney disease.

Insights from animal models

Animal models to study the role of mast cells in renal disease: A general overview

The analysis of MC function in renal diseases has been greatly helped by the use of MC-deficient animal models so far mostly based on the Kit-dependent models: WBB6F1-*Kit*$^{W/W\text{-}v}$ and C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice [56]. As indicated before, because c-KIT is expressed also on other cells within or outside the immune system, the effective implication of MC needs to be verified after reconstitution of MC-deficient mice with in vitro bone marrow–derived MC. When reconstitution completely normalizes the phenotype, this indicates an MC nonredundant function, while partial reconstitution indicates a contributory role of MC. It is also possible that other cells can compensate for the function of MC. Hence, the effective MC contribution may not be revealed in these studies. Another word of caution may come from the fact that mouse or rat experimental kidney diseases can differ from human kidney diseases as MC infiltration is significantly less predominant. This may likely be explained by the fact that CKD in humans can develop over long time spans that can be years, allowing ample time for progressive MC infiltration. Yet, regional contributions from their location in lymph nodes [126] or even systemic contributions of MC activated in some distance [13] in highly inflammatory models may be relevant also in human disease. Likewise, the contribution of MC in the connective tissue surrounding the kidneys has not been investigated in detail. Kidney capsule MC, which are prominent both in normal kidneys and in kidney disease, where shown to get activated in disease [127], and therefore could play an important functional role.

Mast cells in autoimmune glomerulonephritis

Several groups have studied MC function using W/W^{-v} mice in an experimental model of Goodpasture disease initially developed by Masugi [128]. This model, after injection of allo-antibodies directed against the glomerular basement membrane (GBM), involves a heterologous phase of disease development characterized by a rapid, but transient influx of neutrophils that, through FcR- and complement-dependent mechanisms, leads to the initial glomerular injury. The development of an immune response to the injected antibodies by the host promotes the development of the second autologous phase characterized by infiltration of FcR-bearing macrophages and T cells as effectors of glomerular injury and that further promote disease development toward renal failure. The importance of T cell response has been demonstrated in several studies. They suggest a role for CD4 T cell-driven Th1-type responses with development of delayed-type hypersensitivity-like cellular effectors (CD8 T and macrophages) as well as Th1-type Ig isotype production [129]. Defects in regulatory T cells have also been implicated as a potential mechanism of pathology [130]. When hosts are preimmunized with normal nonpathologic antibodies a few days before injection of the anti-GBM antibody, the appearance of the autologous phase can be accelerated. It should be noted, however, that this model does not exactly reproduce human Goodpasture disease as anti-GBM autoantibodies do not derive from a different species.

Interestingly, when studied in MC-deficient mice, the anti-GBM model results revealed quite different outcomes and conclusions. While two studies reported a beneficial role of MC [114,126], another study indicated an aggravating role [131]. The studies showing protective functions of MC found an increased mortality in MC deficient W/W^{-v} mice when compared to WT mice or W/W^{-v} mice reconstituted with MC. The striking pathological feature in both studies was the presence of large subendothelial deposits in W/W^{v} mice, while they were less prominent in WT mice or MC-reconstituted W/W^{v} mice. These deposits appeared early during the heterologous phase of the disease and persisted thereafter. Further analysis in one study showed that glomeruli of W/W^{-v} mice contained increased amounts of fibrin and type I collagen [114]. In agreement with the high amounts of fibrin, W/W^{-v} mice were unable to maintain high tissue plasminogen activator (tPA) and urinary-type plasminogen activator (uPA) activity in urine at the late phases of the disease indicating an important role of MC in these functions. The contribution of MC to fibrin remodeling after injury may therefore represent a key factor to protect against glomerulonephritis development and disease progression. Both studies also found increased amounts of inflammatory macrophages and, in one study [126], increased amounts of T cells infiltrating the glomeruli and interstitial space of W/W^{-v} kidneys. This led the second study to conclude that MC modulate the influx of effector T cells and macrophages to limit inflammation. None of the two studies detected significant MC infiltration in the kidney, but the study by Hochegger et al. clearly showed significant MC infiltration into draining lymph nodes supporting regional immunoregulatory effects by MC [126]. The authors indicated an interrelationship between MC and regulatory T cells (Tregs) previously reported to protect against the disease [126]. Additional studies clearly presented evidence for a MC/Treg crosstalk involving Treg produced IL9 attracting MC to the lymph nodes being necessary to support the immunoregulatory protective functions of Tregs [132]. Interestingly, although Th17 cells have been incriminated in the promotion of anti-GBM GN, in a more recent study a protective function of locally produced IL17 was reported, with MC being producers of protective IL17 [133].

Surprisingly, in the third study, the authors found a disease-aggravating role of MC using a nonaccelerated model of the disease injecting allo-antibodies in two individual doses in the absence of adjuvant and scoring pathology at day 21 [131]. Under these conditions no mortality was observed, but renal function parameters were significantly more deteriorated in WT mice as compared to MC-deficient mice. Further analysis indicated an enhanced dermal delayed (DTH) type hypersensitivity response supporting that MC under these conditions enhance pathologic Th1-dependent effector cells. Immunohistochemical analysis also showed increased expression of integrins in glomeruli of WT mice, which could be responsible for the increased infiltration of macrophages observed under these conditions.

Although the three studies reach somewhat different conclusions, they may actually reveal the delicate balance of the inflammatory reaction as well as the high versatility and sometimes contrasting function of the MC as an effector cell. As noted previously, MC may be able to sense the particular pathophysiological context such as the strength and kinetics of disease development [49]. Thus, in the first study, based on a rapidly developing accelerated disease model, the major MC mediated effect was a direct protective fibrinolytic activity. In the second study, which according to the kinetics of mortality was based on a more slowly developing, yet still accelerated disease model, the major effects were protective immunoregulatory functions. In the third study using a more chronic non-accelerated disease model, the MC-mediated responses rather promoted a Th1 type immune response and hence enhancement of disease activity. Remarkably, even in the accelerated model, examination of MCPT-4, a granule stored chymase [23], revealed that this enzyme promoted the enhancement of disease indicating that, while the global action of MC is disease protective one particular mediator might act differently [127]. In particular, MCPT-4 enhanced proinflammatory cytokine production and the inflammatory cell infiltrate and glomerular deposition of collagen. As a possible mechanism, MCPT-4 was shown to act as a tissue generator of the proinflammatory and profibrotic acting peptide Ang II. Although no MC and MCPT-4 were found in the kidneys, MCPT-4 was present in kidney capsules and induction of anti-GBM GN induced the degranulation of kidney capsule MC.

Together, these studies reveal that MC are able to exert a spectrum of responses ranging from protection to aggravation in a similar disease model with timing and strength of the insult being important parameters. One can speculate that while the immediate action of MC is destined for repair, more progressive disease may also reveal a detrimental action of MC responses as they may become inadequate if not downregulated. In addition, these studies also showed that MC contain different type of mediators that can be either pro- or antiinflammatory.

Another form of rapidly progressive GN is anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). AAV is an autoimmune disease, involving T and B cell autoreactivity to neutrophil lysosomal enzymes proteinase-3 and myeloperoxidase (MPO), resulting in the development of focal necrotizing GN. A focal necrotizing GN resembling human AAV can be induced by immunizing mice with MPO followed by the injection of a subnephritogenic dose of ant-GBM antibodies in order to locally recruit and degranulate neutrophils thereby depositing MPO in the glomeruli. When AAV was induced in WT or MC-deficient W^{sh}/W^{sh} mice, the authors again found that MC can be protective as MC-deficient mice exhibited more anti-MPO $CD4^+$ T cells, enhanced dermal delayed-type hypersensitivity responses to MPO, and more severe focal necrotizing GN [134]. The protective action of MC was confirmed after reconstitution of MC-deficient mice with MC. Further studies showed that MC recruited to the draining lymph nodes were able to secrete IL10 thereby increasing the number of Tregs, which counteracted proinflammatory T cell populations notably Th1 and Th17 cells. Interestingly, the protective function of MC could even be further increased when limiting at the same time MC proinflammatory functions notably by blocking their activity of mediator release by degranulation with cromolyn, while maintaining IL10 production [135].

Systemic lupus erythematosus (SLE) is another autoimmune disease that can lead to GN and predominantly affects young women. It is characterized by the presence of autoantibodies against nuclear cell antigens and almost specifically against double strand-DNA. Circulating immune complexes deposit in various organs and tissues such as skin, joints, the nervous system and kidney. The resulting inflammation leads to various clinical manifestations, which include besides dermatitis, arthritis, neurologic disorders and GN. Renal involvement represents a critical for the outcome of SLE since they could lead to renal failure [136]. The involvement of MC in at least some of SLE manifestations, such as nephritis, has been suspected early on [137]. Indeed, renal tubulointerstitial MC shows an about 10-fold increase in SLE nephritis patients compared to healthy individuals and correlate with fibrosis [138,139]. However, no correlation was found between MC numbers and clinical outcome [140], indicating that although MC may contribute to the disease in this organ they are not the only factor involved. Here also, the use of MC-deficient mice in an experimental model of pristane-induced lupus nephritis has allowed to shed some light on the possible implication of MC. Analysis of MC-deficient animal models of lupus such as the pristane-induced model or aged Lyn KO mice crossed with MC-deficient W^{-sh}/W^{-sh} mice showed protective roles of MC [141] while in

the spontaneous Lyn KO model no notable role of MC became apparent [142]. In the first model, although no differences in proteinuria were noted, the absence of MC in W/W^{-v} mice led to a diffuse proliferative GN instead of focal segmental GN, and to somewhat more important immune deposits in the glomeruli. However, no MC knock-in study has been performed leaving open the possibility of MC independent effects. Nonetheless, it was relatively interesting to observe that both in anti-GBM GN and pristane-induced lupus GN a striking pathological feature in absence of MC was an enhanced immune deposition in the glomeruli, indicating that one of the MC beneficial promoted action is the inhibition of glomerular immune deposits and/or their enhanced clearance. Although the second study [142] did not notice a significant role of MC, it revealed an important role of closely related basophils and auto-IgE antibodies, which via an autocrine mechanism of released PGD2 and expressed PTGDRs induces the externalization of CXCR4 on basophils, both in humans and mice, driving accumulation in secondary lymphoid organs, where they amplify autoantibody production [142,143].

Mast cells in acute kidney injury

Acute kidney injury (AKI) has increased steadily and is reported to occur in 2%–7% of all hospitalized patients [144]. AKI is characterized by an abrupt decrease in kidney function as measured by increases in serum creatinine. It can have multiple causes ranging from infectious or toxic insults, hypotension, ischemia etc. Despite therapeutic progress, AKI continues to be associated with high morbidity and mortality, particularly in patients admitted to intensive care units (ICU). For survivors, there are chronic consequences with a high risk of developing CKD. AKI associates with intrarenal and systemic inflammation. Hence, the understanding of the inherent cellular and molecular mechanisms is important to identify new therapeutic strategies [145]. MC are important players of the inflammatory response and their implication has been investigated in two experimental model systems. In the first model, Summers et al. tested their role in cisplatin-induced AKI using W^{-sh}/W^{-sh} mice [146]. They found that deficiency of MC attenuated renal injury, reduced serum levels of TNF and intrarenal levels of leukocyte chemoattractants, thereby limiting recruitment of leukocytes to the inflamed kidney. The phenotype was reversed to the one observed in WT mice after reconstitution of MC-deficient mice with in vitro-grown MC, but not when they were deficient in TNF. Hence, TNF, which can be rapidly released from MC granules, was a major mediator of MC injury following the toxic insult with this anti-cancer drug. The role of MC was also examined in a model of renal ischemia reperfusion injury (IRI). Following renal IRI one observes a rapid infiltration of immune cells, in particular neutrophils, favored by the disrupted and damage-activated endothelium along with substantial release of inflammatory cytokines and chemokines. This acute phase of the inflammatory response (up to 48 h post-IRI) is then gradually replaced by a late injury phase where one observes other major immune effectors, notably T lymphocytes and monocyte-derived macrophages. Late-phase immune modulation may be crucial for repair and eventually lead to regeneration of the injured tissue, but conversely can also promote an inappropriate immune response leading to organ atrophy and irreversible fibrosis [147]. The role of MC was examined both in an acute (involving the clamping and reperfusion of both kidneys) and a long-term model (clamping and reperfusion of a single kidney) in RMB mice [57,148]. The latter is a KIT-independent model of MC deficiency where MC are depleted by the administration of diphtheria toxin (DT). In control PBS-injected mice, after renal IRI, one observes systemic activation of MC as evidenced by the significant rise of MCPT-1, a murine MC-specific chymase, in the blood. MC activation proved to be detrimental as administration of DT prior to IRI to deplete MC resulted in improved renal function with diminished local inflammatory cytokine/chemokine levels and reduced pathologic neutrophil recruitment to the kidneys [148]. Furthermore, although not completely protected, MC–depleted mice displayed less organ atrophy and fibrosis than did PBS-injected mice during the chronic phases (2 and 6 week post-IRI). Interestingly, however, MC ablation after the acute phase of IRI had no impact on organ atrophy, tubular necrosis or fibrosis, suggesting that MC are detrimental during the acute inflammatory phase promoting subsequent fibrosis development, but not during the chronic phase of the disease. A more recent study investigated the role of MCPT-4 chymase using the acute IRI model [13]. Here, the opposite result was obtained as this chymase proofed to be protective. The authors showed that WT mice had less infiltrating neutrophils than MCPT-4-deficient mice. This was explained by the fact that MCPT-4 and human chymase cleaved adhesion molecules crucial for neutrophil extravasation including CD162, CD54, P- and *E*-Selectin. MCPT-4 was also able to directly dampen neutrophil activation, by a so far unknown mechanism. Together, these results again show that MC can release simultaneously both pro- and antiinflammatory compounds. Released MCPT-4 chymase has a potent antiinflammatory effect that controls neutrophil extravasation and activation, thereby limiting associated damage. The simultaneous action of pro- and antiinflammatory mechanisms represents a well-known feature of the inflammatory response, which is governed by both proinflammatory and antiinflammatory systems [149].

Mast cells in renal fibrosis

As indicated above, many studies have noted an increase of MC in kidneys that was correlated with the progression of fibrosis and decrease of renal function. However, direct evidence that MC participate in renal fibrosis has not been provided although several observations in human disease pointed to a fibrogenic potential of kidney infiltrating MC. This includes the expression of type VIII collagen mRNA expression by MC in diabetic nephropathy [117], the expression in MC of fibroblast-proliferative factor FGF in IgA nephropathy [150] and the presence in MC of profibrogenic TGF-β1 in progressive renal disease [151]. TGF-β1 and TGF-β1-initiated Smad signaling pathways are key effector mechanisms of tubulointerstitial fibrosis development [152–154] inducing extracellular matrix (ECM) production and myofibroblast development.

Unexpectedly, however, two studies using experimental animal models reported that MC actually ameliorate renal fibrosis. Miyazawa et al. utilized the puromycin aminonucleoside-nephrosis (PAN) model in MC-deficient W^s/W^s rats [155] promoting a diffuse tubulointerstitial fibrosis with ECM expansion and increases in interstitial macrophages and T cells over a period of several weeks [156]. Following puromycin administration both disease groups developed proteinuria and renal failure at about 6 weeks with a concomitant increase in blood urea nitrogen. While no differences were observed in these parameters between the two groups of rats, fibrosis development as quantified by histology and hydroxyproline levels was more severe in W^s/W^s rats when compared to WT rats. Furthermore, profibrotic cytokines IL-4 and TGF-β1 mRNA levels were higher in the MC-deficient rats. Although the authors did not verify their findings in reconstitution experiments the authors indicated as a possible mechanisms heparin secreted by infiltrating kidney MC, which is known to inhibit TGF-β1 production by renal fibroblasts in vitro. A similar protective role was also seen in the unilateral ureteral obstruction (UUO) model when MC-deficient W/W^{-v} mice were examined. UUO, which involves ligation of one ureter, is a rapidly evolving model of renal tubulointerstitial fibrosis reproducing the different stages of obstructive nephropathy [157]. This includes cellular infiltration, tubular proliferation and apoptosis, epithelial-mesenchymal transition (EMT), myofibroblast accumulation, increased extracellular matrix (ECM) deposition and tubular atrophy. When applied to MC-deficient W/W^{-v} mice, they revealed within 3 weeks significantly increased tubular injury and fibrosis as well as a decrease in cadherin expression and increase in alpha smooth muscle actin when compared to WT mice [158]. Reconstitution of mice with MC restored the protective effect, effectively demonstrating the implication of MC. Although no significant increases in MC numbers were noted, WT mice showed a diminished macrophage and T cell infiltrate and lower TGF-β1 levels as compared to MC-deficient mice. The authors concluded that MC protected from renal fibrosis by diminishing infiltration with profibrotic cells. Surprisingly, two other studies reached the opposite conclusions, indicating that MC aggravate fibrosis. Veerappan et al. reported that WT rats or mice after UUO developed a significant higher fibrosis score than rats treated with the MC stabilizer sodium cromoglycate or MC-deficient W/W^{-v} mice [159]. This was accompanied by heavy MC infiltration on day 14 UUO rat kidneys. As judged from perfused kidneys they released active renin and generated local Ang II promoting increased vascular resistance and fibrosis. In support, fibrosis development in UUO rats was attenuated by the Ang II receptor blocker Losartan. A second study performed in MC-deficient W^{sh}/W^{sh} mice came to similar conclusions showing that fibrosis and accompanying inflammatory parameters were enhanced in WT mice when compared to MC-deficient mice [160]. In this study, while significant MC infiltration was observed in draining lymph nodes, no MC infiltrate was observed in kidneys although mediators such as tryptase and chymase were detected. However, kidney capsule MC and/or systemic MC activation was not investigated in this study. Another study testing the role of MC specific MCPT4 chymase in MCPT4-deficient mice revealed a protective function of this protease in UUO [161]. Given that in the anti-GBM model (see above) MCPT4 had an aggravating profibrotic role through the generation of local Ang II in WT mice, this was surprising. However, it seemed that in UUO, local Ang II generation via MCPT4 may be insignificant over the extra-tissular activation of the systemic renin–angiotensin system. This was supported by the evaluation of Ang II production, which was similar between the two strains. Yet, MCPT4 was able to degrade the extracellular matrix fibronectin, thereby preventing recruitment of proinflammatory cells such as macrophages and T cells, which led to the protective function of this enzyme. A closely related model of partial unilateral ureteral obstruction (pUUO) in newborn mice was also investigated in MC- and MCPT4-deficient mice [162]. The pUUO model reproduces more closely the situation of kidney fibrotic lesions in human obstructive nephropathy, which develop more slowly than after complete UUO. Measurement of kidney length and volume by magnetic resonance imaging (MRI) as well as postmortem kidney weight revealed hypotrophy of operated right kidneys (RKs) and compensatory hypertrophy of left kidneys, which was attenuated in MC-deficient W^{sh}/W^{sh} mice and intermediate when mice lacked MCPT4 chymase. As α-SMA expression, a marker of epithelial-mesenchymal transition (EMT), was higher in WT mice, the authors concluded that MC favored the initial stages of fibrosis development in this long-term model and that this was at least partially dependent on MCPT4

chymase. The possible long-term contribution was also investigated following induction of ischemia–reperfusion injury (IRI) in Kit-independent RMB mice where MC deficiency can be induced after injection with diphtheria toxin [57]. Interestingly, as already indicated above, in this model when MC deficiency was induced past the acute phase of injury no significant MC effects on fibrosis were noted, while MC clearly promoted fibrosis when MC deficiency was induced before the acute phase [148]. This may relay to the relative absence of MC in mouse kidneys even in the progressive phase, while during the acute phase clearly systemic MC activation as judged by increased levels of MCPT-1 chymase were noted in the first hours after reperfusion.

Taken together, these studies again reveal a highly complex picture regarding the contribution of MC as both protective and aggravating functions are reported with different mechanisms. It again shows that the pathophysiological context (complete or partial UUO) may be important. Similarly, while in the anti-GBM model local Ang II generation by mMCPT4 appears to be important, this is not the case in the UUO model enabling at the same time to observe the impact of other physiological functions of chymase such as the degradation of fibronectin. Differences exist also concerning the evaluation of infiltrating MC. While one study reported high numbers of MC in the range of 125–300 cells/mm^2 [159] largely exceeding those reported for human renal diseases [6] most other studies reported modest MC infiltration in various model systems.

Positive actions of mast cell mediators in kidney disease

The above studies in humans and mice have highlighted the importance of inflammatory processes in renal disease. It functions in a highly orchestrated and timed manner involving both pro-inflammatory, but also antiinflammatory mechanisms to achieve repair of lesions, promote tissue remodeling and restore tissue homeostasis [149]. The repair and remodeling process usually leads to the development of scars and limited fibrosis. Under certain circumstances it may also allow replacement of lost cells and regeneration of the renal parenchyma [103,105]. Yet, when the targeted destruction and assisted repair mechanisms are not properly phased, inflammation will lead to persistent tissue damage.

MC represent integral actors of the inflammatory response in renal diseases. While mostly known for their proinflammatory actions, evidence now exists indicating that they also have beneficial roles by participating in antiinflammatory responses and tissue repair. In the following, a variety of the positive actions by which MC can contribute to this repair response will be resumed.

Fibrinolytic system

Fibrin clots built as an immediate response to tissue injury, but need to be removed during the tissue remodeling phase. MC are important cellular actors of the fibrinolytic process by producing besides heparin (see below) tissue plasminogen activator tPA, a major activator of plasminogen, which converted into plasmin dissolves fibrin clots [36]. While tPA is produced constitutively by MC they also synthesize its natural inhibitor PAI-1 (plasminogen activating inhibitor-1), however, only upon stimulation [36]. This suggests that at least in the acute inflammatory phase tPA may be inhibited by MC. However, experimental evidences in the anti-GBM GN model have clearly demonstrated that the MC net contribution is the dissolution of subendothelial fibrin deposits in vivo [114]. These experiments have also shown that MC contribute to the maintenance of high levels of uPA and tPA in urine during the later phase of GN development to support subendothelial fibrin dissolution.

Heparin

Heparin is evolutionary conserved even in organisms that do not have a coagulation system. Besides its anticoagulation activity it participates in many other antiinflammatory activities by its capacity to bind to many inflammatory chemokines, cytokines complement factors growth factors, thereby preventing them for example to interact with their respective receptors [163]. Consistently, oral heparin-like compounds like oral pentosan polysulfate have been shown beneficial effect in the treatment of glomerulosclerosis [164]. Of note, MC are the unique producers of heparin in the human body. This highly sulfated glycosaminoglycan, which is released from cytoplasmic granules by degranulation [11,23]. Concerning its anticoagulant activity, while heparin does not break down existing fibrin clots, it complements the body's natural fibrinolytic system, i.e., the plasminogen activating system by preventing reclotting. Hence, during the repair phase following tissue inflammation and especially in the course of renal diseases, heparin-mediated antiinflammatory properties of MC might complement the other aforementioned fibrinolytic actions of MC.

MC proteases

MC secrete a variety of proteases such as chymase, tryptase and MC carboxypeptidase A (MC-CPA) uniquely produced by these cells. Clearly, the role of these enzymes has to be considered in the multistep process leading to CKD [23,165]. For example, chymase has been described as an important local producer of Ang II, independent of the classical RAS. Of note, MC may also contain active Renin themselves and as such contribute also locally to the RAS system. The resulting product, Ang II, has been known for many years as a major contributor to renal diseases and inhibition of its action can slow down CKD development and progression [166]. Similarly, tryptase has been reported to activate fibroblasts via PAR-2 receptors, thereby contributing to fibroblast proliferation and synthesis of collagen [23]. Tryptase was also reported to directly activate profibrotic TGFβ that is produced as a latent inactive complex in the extracellular matrix [77]. However, mounting evidences also highlight protective functions of these proteases again suggesting that the appropriate balance and timing of their action is a critical parameter. Thus, related to renal disease pathology, albeit MC produce endothelin-1, a peptide with potent vasoconstrictor activities, they also release at the same time chymase and MC-CPA that have been shown to degrade it [68,167]. Endothelin-1 is produced in high amounts in various nephropathies and endothelin receptor antagonism has protective effects by reducing renal vascular fibrosis [107]. Chymases and tryptases have also been reported to cleave and inactivate several types of proinflammatory cytokines including IL-5, IL-6, IL-13 and TNF or the chemokines eotaxin and RANTES [165]. Similarly, proinflammatory neuropeptides released from nerve endings can be neutralized by the action of chymase and tryptases [165]. Thus, MC proteases may participate also in the downregulation of an acute inflammatory response. Hence, although MC as a whole release a wide array of products that support AKI after ischemia reperfusion injury, recent data show that concomitantly released MCPT4 chymase participates to control excessive infiltration of pathologic neutrophils [13]. MC proteases also play an important role in the tissue remodeling process as MCPT4-deficient mice exhibit a slight increase in extracellular matrix production [23]. Chymases and tryptases can contribute to matrix destruction by directly cleaving ECM proteins fibronectin and non-helical collagen. Hence, MCPT4 chymase was able to limit pathologic T cell infiltration and hence fibrosis development after complete UUO by its ability to cleave the ECM protein fibronectin [161] yet in the more slowly developing long-term model of partial UUO this effect was no more visible and MCPT4 rather contributed to promote fibrosis development again indicating that the pathophysiological context is important [162].

Matrix metalloproteinases

Remodeling of the extracellular matrix (ECM) is a key event in the progression of CKD but also in reversal of CKD in a regenerative perspective. Degradation of ECM proteins is necessary to allow inflammatory cell infiltration to the injurious site and is also required in the repair phase to limit excessive deposition of ECM proteins in the interstitial space and in glomeruli vessels. The degradation of accumulated ECM proteins, besides the aforementioned MC proteases, is largely mediated by matrix metalloproteinases (MMP). MMP themselves, are under control of proteases such as plasmin converting the proforms into active forms as well as of specific tissue inhibitors of metalloproteinases (TIMP). Because of their multiple actions, MMP may exert different and even opposite roles during the various phases of renal diseases with the major challenge being temporal control of MMP activity during disease development [168]. A direct demonstration of the beneficial potential of MMPs in renal disease was obtained in experimental anti-GBM GN where MMP9 has a protective action through its profibrinolytic activity [169]. Similarly, in acute kidney injury induced by folic acid, MMP9 deficiency was shown to increase apoptosis and delay recovery of renal function. The effect was attributed to an increased bioavailability of apoptosis-protecting SCF through increased generation of soluble SCF by MMP9 from a membrane precursor [109]. Whether the generation of soluble SCF also attracts "protective" MC has not been investigated in this study. MC can directly produce certain MMP, such as MMP2 (gelatinase A) and MMP9, but are also able to activate their proforms. Mice deficient in MCPT4 have almost exclusively inactive pro-MMP9 in tissue extract as compared to WT mice [170]. A major action of MC may result also from their ability to inactivate endogenous inhibitors. MC chymase can directly degrade endogenous tissue inhibitor of metalloproteinase-1 (TIMP-1) [171]. It bears noting that the actions of chymase differ from those of β-tryptases, which are not effective activators of MMP9, although their ability to activate pro-stromelysin/MMP3 provides a potential means of activating MMP9 indirectly via MMP3 [165].

Histamine

Histamine is an important inflammatory mediator majorly produced by MC well known for its effect in allergies. Although early studies have reported an association of renal disease with atopy and elevated IgE levels, this was

not a consistent observation [6]. Surprisingly, experimental evidences suggests that histamine may even have an antiinflammatory function in the experimental anti-GBM GN model in rats [172]. Direct administration of histamine led to significant dose-dependent reduction in proteinuria compared to control-injected group and markedly decreased the number of crescentic glomeruli and macrophage infiltration of the glomeruli. Receptor agonists, like the H2/H4 agonist (Dimaprit) mimicked the effects of histamine. H1 mepyramine or H2 (ranitidine) antagonist did not reverse the protective effect of histamine. Based on this, it was proposed that histamine may alleviate renal injury in anti-GBM glomerulonephritis by its effect on H4 receptors, which are majorly expressed on immune effector cells, where it is involved in multiple inflammatory, autoimmune an immunoregulatory processes [173]. In this line, monocytes activation of H4 receptors was reported to downregulate CCL2 chemokine production [174]. On the other hand, H4 receptor may also play a negative role in diabetic nephropathies as it was overexpressed on renal tubular cells in diabetic rats, where it may have a direct effect on tubular reabsorption of blood sugar and an indirect action on renal tissue architecture via inflammatory cell recruitment [175]. However, more studies are necessary to further substantiate the histamine-mediated antiinflammatory action in renal disease.

Immunoregulatory properties of mast cells

MC are able to produce a wide range of cytokines with immunoregulatory properties. As described above in different inflammatory set-ups, MC besides promoting inflammatory responses can also have antiinflammatory and immunosuppressive functions. In particular, they were important in the crosstalk with regulatory T cells (Treg) to establish peripheral tolerance in a mouse skin allograft model [97]. Experimental evidences have shown a renoprotective effect of Tregs in several models of renal pathology including anti-GBM GN, adriamycin induced nephropathy and ischemia–reperfusion injury [126,130,176–179]. They are also important effectors to allow stable kidney transplant recipients with donor-specific cytotoxic-hyporesponsiveness [180]. Further studies in the anti-GBM model showed that it is Treg-produced IL9, which attracts MC to lymph nodes to establish a protective MC/Treg crosstalk [132].

MC also have the ability to downmodulate T cell responses via the production of soluble mediators. Thus, MC also produce IL-10, with multiple antiinflammatory actions including the downregulation of T cell responses, induction of Treg, downregulation of adhesion molecule expression etc [11]. Protective IL10 production by MC has been directly been imaged in a model of severe contact hypersensitivity [56]. In renal disease the renoprotective effect of IL-10 in the downregulation of the inflammatory responses in both acute and chronic renal experimental diseases has been demonstrated in knock-out mice [181–183]. MC besides IL-10 also produce TH2 polarizing cytokines IL-4 and IL-13, which may shift the effector T cell balance. Both of these cytokines have also been implicated in promoting the generation of "alternatively" activated macrophages that have been previously classified as M2 macrophages as compared to classically activated M1, although with the advent of single cell RNAseq the heterogeneity in this population becomes more and more evident. M2 macrophages have important antiinflammatory functions suppressing immune responses and promote tissue remodeling. Hence, they play important roles in regulating homeostasis, kidney injury, repair, and tissue fibrogenesis [184]. Presently, however, information on the crosstalk with MC is sparse, albeit it has been reported that MC tryptase can promote the polarization toward a profibrotic phenotype [185].

Growth factors

Vascular endothelial growth factor (VEGF) is regarded as an important regulator of neovascularization in many physiological and pathophysiological processes. It is also one of the most potent vascular permeability factors yet described, with a molar potency that exceeds histamine 50,000-fold [186]. CKD is characterized by tubulointerstitial hypoxia and peritubular capillary rarefaction and the importance of VEGF in renal recovery has been demonstrated in various experimental and human renal diseases [186–188]. MC are important producers of VEGF and hence may contribute to orchestrate neovascularization in kidneys [186,189]. Another important growth factor in renal protection is hepatocyte growth factor (HGF), which has antifibrotic activities. Initially discovered for its implication in liver regeneration it was also shown to have potent antifibrotic activity, a function that can be attributed to its ability to inhibit TGF-β1–mediated signaling. HGF improved chronic injury in a variety of experimental settings and treatment with a recombinant form either at disease onset or after establishment of disease attenuates renal fibrosis [190]. It was shown that MC are important stimulators of HGF activity through the release of heparin necessary for its function likely by promoting receptor oligomerization [191,192].

Lipid derived mediators

MC generate three arachidonic acid derived lipid eicosanoids: prostaglandin PGD2, leukotriene LTB4 and LTC4, the parent molecule of the cysteinyl leukotrienes (cysLTs). Although not exclusively produced by MC these mediators have powerful functions mediated through activation of specific cognate G protein coupled receptors including in the kidney where they are involved in the pathology of various renal diseases [193,194]. Yet, antiinflammatory action has been ascribed to 15-deoxy-d(12,14)PGJ2 (15-dPGJ2), an in vivo metabolite of PGD2. When bound to peroxisome-proliferator-activated receptor-γ it induces apoptosis of infiltrating neutrophils and macrophages recruited to clear these apoptotic cells, thereby supporting the clearance of the inflammatory infiltrate [30,31]. In a model of renal IRI in rats 15-dPGJ2 improved renal function (plasma creatinine levels) and reduced the histological signs of renal injury. At the same time 15-dPGJ2 markedly reduced the expression of inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 expression during reperfusion and activation of nuclear factor (NF)-kappaB in renal cells [195]. Thus, certain metabolites of lipid-derived mediators may be useful antiinflammatory agents and/or regeneration actors that enhance the tolerance of the kidney against the renal dysfunction and injury.

Concluding remarks

MC have now been established as important effectors in kidney disease. While only sparsely present in kidneys of healthy subjects, they are an integral part of the inflammatory cell infiltrate appearing during disease development, albeit little information still exists regarding their action in the long term. Besides their local presence several studies have also highlighted possible actions of MC in the kidney capsules where one can observe significant MC activation. Immunoregulatory functions have also been ascribed to MC infiltrating the draining lymph nodes. In highly inflammatory experimental models such as IRI systemic MC activation and action has also become evident. Likely due to their negative roles they play in allergy they have been regarded as disease aggravating effector cells, although this was mostly based on correlative data. Recent experimental evidences, however, impose a subtler view with regard to their involvement in renal disease with numerous positive roles of these cells. This rejoins increasing data from the literature recognizing many positive functions of MC in host defense mechanisms, detoxification and inflammatory diseases [11, 12]. Yet, as clearly evidenced in this chapter, their role in disease have revealed a highly complex picture. Thus, both disease protective and disease aggravating functions can be demonstrated in the same experimental renal disease model. This suggests that environmental cues such as reagents, housing conditions, dosage or kinetic parameters of disease development may play an important role, likely because MC are able to sense their environment to undertake action. Strain-specific differences have also become apparent as suggested by data obtained in c-Kit dependent MC deficient models such as W/W^{-v} and W^{sh}/W^{sh} mice that show additional abnormalities including in the hematopoietic compartment [158,159]. New MC-deficient animal models have now become available and have been started to get investigated.

One of the prime functions of MC is to serve as sentinels that participate in the initial inflammatory response by rapidly releasing mediators to counteract an injurious event by rapid mediator secretion. When appropriately regulated, the initiated response funnels smoothly in an antiinflammatory response and repair phase to restore homeostasis. Besides providing crucial mediators for these various phases, MC via their ability to sense the inflammatory response through numerous expressed receptors they importantly participate in its regulation by the timed secretion of many immunomodulatory products (Fig. 8.3). These impact on the activity of other immune effectors including T cells and macrophages that participate in the resolution of inflammation for example by the provision of growth factors and through the degradation of accumulated ECM proteins during the repair phase. Therefore, we can postulate that the prime role of MC is to orchestrate remedial strategies to counteract the injurious event and to participate in repair and regenerative responses. However, it should be noted that while beneficial roles of MC have clearly emerged, chronic or excessive stimulation or defective regulatory processes may shift the balance in the opposite way. This is exemplified by data showing opposite outcome in similar animal models of kidney disease. Therefore, the prime interest of any therapeutic activities is to favor the positive actions and to suppress at the same time the negative activities.

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PART III

Stem cells in regenerative processes

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CHAPTER

9

Regeneration and replacement of endothelial cells and renal vascular repair

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Introduction

The kidney receives a large proportion of the cardiac output. A complex and integrative vascular network exists to accommodate this large volume of blood flow and fulfill the primary functions of filtration and reabsorption necessary to remove toxins and regulate fluid and electrolyte balance. During progressive chronic kidney disease or in response to acute kidney injury, renal blood flow is compromised. Impairments in the renal microcirculation may promote damage to renal parenchymal tissue, while tubular injury also impacts the vasculature [1, 2].

Given the strong association between impaired renal microcirculation and disease states, significant attention has been directed toward understanding renal vascular repair. Endothelial progenitor cells (EPCs) have been studied in different cardiovascular models, including preclinical models of kidney disease. However, as this field of study has evolved over the last 20 years, it has been hampered by inconsistent definitions surrounding the term EPC. Originally coined to describe a circulating cell population derived from bone marrow that displayed homing, localization, and enhancement of vascular repair of ischemic tissue via formation of new vessel formation [3], it is now recognized that multiple different cell types capable of providing beneficial angiogenic effects in disease were inappropriately broadly identified and assigned the term "EPCs," when in fact only a small unique subset of cells truly possessed vessel-forming potential [4]. Recently, incorporation of more precise nomenclature, encompassing careful attention to cell phenotype and function, has permitted agreement on consensus criteria to distinguish between these cell types and to better understand their mechanism of action [5]. These criteria when widely applied will potentially move the field forward into the clinical realm with clear indications for which type of cell therapy is required in a disease-specific manner to yield regenerative vascular outcomes.

This chapter attempts to provide relevant background regarding vascular injury and impaired regenerative responses in the setting of either acute kidney injury (AKI) or chronic kidney disease (CKD). We provide an updated discussion on different types of cells previously classified broadly as EPCs and discuss potential interactions of these cells in vascular repair. Using this framework, we present a hypothesis by which the activity of intrinsic vascular progenitors represents a central element of disease progression. Finally, we summarize evidence that cells with vasculoreparative properties may represent potential therapies in disease states.

Endothelial impairment in acute and chronic kidney injury

In the acute care setting, reductions in renal blood flow brought about by increased vascular tone or reduced cardiac output may predispose patients to ischemic AKI. *Re*-establishment of renal blood flow by increasing cardiac output, renal vasodilators, or fluid therapy are considered strategies to mitigate AKI. However, if hypoxia is

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prolonged, tissue injury can develop to a point where corrective therapies become ineffective at mitigating injury [6]. A potential explanation for this lack of response is revealed in the concept of the extension phase of AKI proposed by Molitoris and Sutton [2]. This concept places endothelial cell injury at the center of the AKI process and proposes that tissue hypoxia promotes endothelial dysfunction directly or indirectly through mediators released from adjacent epithelial cells. In humans, evidence from biopsies of patients following renal transplant suggests that renal capillary damage correlates with the subsequent development of delayed graft function [7]. Endothelial dysfunction can lead to heterogeneous disruption in perfusion, triggering a cycle of tubular injury and inflammation as hallmarks in the development of AKI [8].

Engagement of the endothelium to limit perfusion is likely due to a combination of different activities. Microvascular endothelial swelling in combination with interstitial edema could predispose the development of microvascular congestion [9]. Disruption of cell–cell contact or the loss in glycocalyx may result in the loss of barrier function [10], which may activate coagulation cascades and exacerbate edema, potentially leading to areas of focal ischemia. Evidence of leukocyte accumulation in cadaveric kidneys of patients with AKI [11] has also been identified in numerous animal models of ischemic or septic AKI along with rouleaux formation. Turbulent or even retrograde blood flow has been identified in postglomerular vessels following ischemic or septic injury [1, 9, 10, 12–16]. Enhanced leukocyte adhesion may result from a rapid upregulation in surface adhesion molecules on the endothelium such as P-selectin [17–19] or ICAM-1 [1, 20, 21]. Adhesion has also been hypothesized to be mediated by the exocytosis of Weibel-Palade bodies from the endothelium [22]. Blockade or inactivation of endothelial adhesion complexes has been shown to reduce the severity of AKI in both experimental models and the setting of renal transplant [9]. Disruption of the congestion by hydrodynamic retrograde perfusion has been shown to enhance recovery from AKI in rats [12]. Experimental interventions designed to relieve impaired hemodynamic responses by influencing the nitric oxide pathway or prostacyclin activity attenuate renal injury (summarized in [6]), and new pharmacological agents which target mitochondrial cardiolipin alleviate endothelial mitochondrial damage and dramatically reduce the degree of renal inflammation following renal ischemia reperfusion [23].

The restoration of renal blood flow is important in the recovery of renal function following AKI. Endothelial repair may be inefficient, and sustained alterations in renal vascular structure and function may be persistent. Evidences from our laboratory and others have documented a reduction in peritubular capillary density of approximately 30%–50% in experimental models of AKI [24]. This capillary loss has been proposed to exacerbate renal hypoxia, widely regarded as a strong positive regulator of interstitial fibrosis, and is a common feature in virtually all models of CKD. Interestingly, a strong negative correlation between capillary density and interstitial fibrosis has been reported in animal models as well as in biopsies from patients with CKD [25–29]. Once initiated, renal fibrosis can result in further damage to the renal interstitium and exacerbate local hypoxia, resulting in a positive feedback to promote progressive CKD (Fig. 9.1). Because of this, there is significant interest in understanding the process of capillary loss and the benefit of potential strategies which can prevent capillary loss or promote vascular repair.

The mechanisms leading to capillary rarefaction following AKI or in CKD are not clearly defined. Careful studies by Ehling et al. have demonstrated that reductions capillary density progress slowly for up to 2 weeks following I/R injury in mice [30]. Our group published evidence of endothelial mesenchymal transition (EndoMT) for up to several weeks following AKI in rats and suggested that this may represent an important factor contributing to vascular dropout [31]. Peritubular capillary EndoMT has been also been reported in models of unilateral obstruction and diabetic nephropathy [32] and in response to a persistent pharmacological reductions in NOS activity absent the presence of hypertension [33]. Although the loss of capillaries is common in the setting of acute and chronic kidney disease, it is interesting that the loss of vessels appears permanent following recovery from acute reversible injuries. This persistent impairment suggests that vasculo-reparative potential of the kidney is minimal. The remainder of this chapter focuses on the degree to which "EPCs" may underlie failed or inefficient vascular recovery in kidney, or the degree to which "EPCs" may be employed as a potential therapy.

Progenitor cells for repairing resident vascular endothelium

Overview of cells that repair or regenerate vascular endothelium

The delivery of blood to a tissue is related to age, overall state of health, activity, disease, and numerous systemic and local intrinsic signals that determine overall blood perfusion. The density of blood vessels in the tissue is one obvious critical determinant that has been well studied [34]. Angiogenesis is the formation of new blood vessels from the endothelial intima of preexisting vessels. During embryogenesis, the first blood vessels are formed from

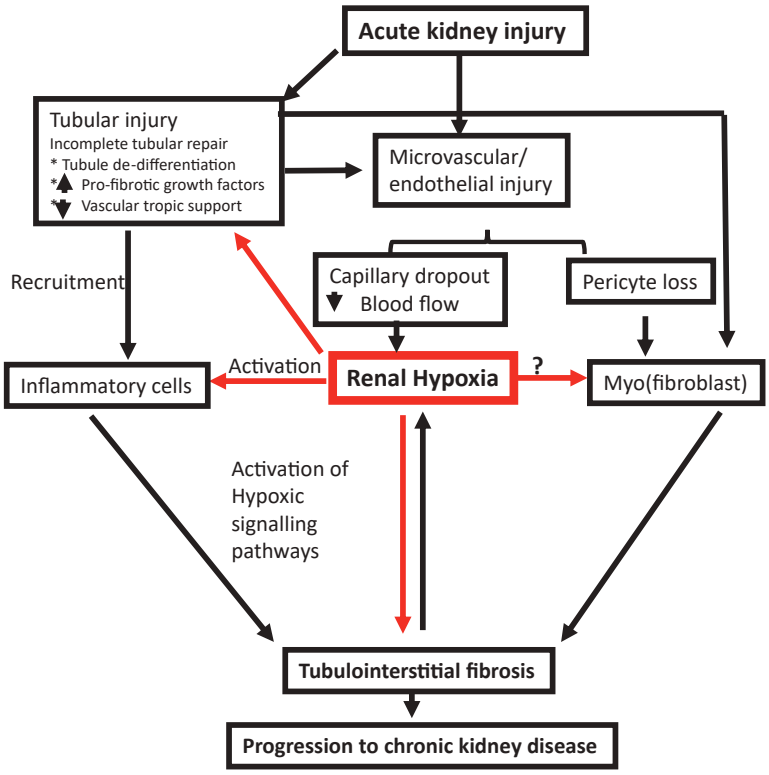

FIG. 9.1 Hypothesized role of renal hypoxia in the progression of acute kidney injury to chronic kidney disease. Acute kidney injury is associated with damage to both tubular and vascular compartments. Tubular injury results in increased expression of profibrotic cytokines and growth factors and decreased expression of vascular factors such as VEGF in tubular cells which may lead to renal microvascular loss. The reduction of peritubular capillaries restricts downstream blood flow from glomerular capillaries and contributes to the development of renal hypoxia, while also being associated with the activation of pericytes toward a fibrogenic myofibroblast phenotype. Hypoxia influences pro-fibrotic and inflammatory activity of tubular and inflammatory cells. Myofibroblast profibrotic signaling is influenced by factors produced from hypoxic tubules or inflammatory cells or may be influenced directly by hypoxia (?). Progressive interstitial fibrosis exacerbates renal hypoxia by increasing oxygen diffusion distance between peritubular capillaries and parenchyma forming a pathological cycle promoting progressive chronic kidney disease. *Reprinted with permission from Ullah MM, Basile DP, Role of renal hypoxia in the progression from acute kidney injury to chronic kidney disease. Semin Nephrol, 2019;39(6):567–580.*

angioblasts that undergo vasculogenesis to form the primitive vascular plexus from which future vessels may sprout via angiogenesis. Arteriogenesis is the remodeling of preexisting collateral arteries to generate larger conductance vessels to replace vessels that have become occluded and insufficient to provide adequate perfusion to a tissue or organ. While angiogenesis is in large part the primary mechanism for increasing perfusion to a tissue by increasing vascular density, this physiologic mechanism does not explain how the resident vascular endothelial intima is repaired when endothelial cells are damaged during aging, diabetes, genetic disorders, infection (particularly systemic inflammatory response), abrupt cessation of blood flow, or trauma. It is in the context of studying the mechanisms of vascular endothelial cell repair and regeneration that the concept of adult vasculogenesis emerged [3, 35, 36], the term "endothelial progenitor cell" (EPC) was coined [3], and numerous clinical trials of EPC were launched in an attempt to promote vascular repair and regeneration in cardiovascular diseases [37–42].

Historical context for defining "endothelial progenitor cells"

Whether or not endothelial cells proliferated at all to regenerate damaged or senescent resident vascular endothelium was not a prominent question prior to 1950, when the robust proliferative response of fetal endothelial cells to form the primary vasculature was thought to be replaced by quiescent endothelium in the adult organism without evidence for a mechanism of replacement. During the 1950s and 1960s, evidence emerged that resident vascular endothelial cells were capable of dividing in areas of vascular intimal damage to repair diseased, defective, or injured cells [43–47]. Yet, questions remained about the potential for circulating cells to be engaged for generating endothelial cells to repair damaged vessels [48, 49].

Asahara and colleagues postulated that some shared cell surface molecules displayed by angioblasts and early hematopoietic progenitor cells may be critical for identifying putative circulating precursors for the endothelial lineage [3]. In their seminal 1997 report, this group identified circulating mononuclear cells that could be enriched via expression of CD34 and CD309, and upon in vitro expansion with angiogenic growth factors, distinct increases in these antigens and Tie2, CD31, and E selectin were noted. As originally predicted by this group, > 90% of the culture initiating cells (CD34 + CD309 +) also displayed CD45 (a marker displayed on all nucleated hematopoietic cells), but this antigen diminished during the culture expansion. Of interest, the plated CD34 + cells displayed colony formation in vitro, and when infused into nude mice with experimentally induced limb ischemia, it demonstrated adhesion to the injured vessels and the implantation was associated with improved perfusion. Hundreds of papers followed with investigators pursuing improved approaches to identifying the cells and accumulation of additional data in preclinical animal models for evidence of vascular repair and regeneration [40, 50–58]. While space limitations deter us from reviewing the massive change in views in the field of EPC study, several consensus articles have illuminated the study of those cells engaged in endothelial repair and regeneration [4, 5, 59–63]. In brief, these review articles provide evidence that cells once collectively called "EPC" are now clearly identifiable as either myeloid angiogenic cells (MACs; and numerous other pro-angiogenic hematopoietic subsets from the bone marrow) or endothelial colony-forming cells (ECFCs; also called blood outgrowth or late outgrowth endothelial cells). These two classes of cells that are engaged in blood vessel repair and regeneration are discussed in the following.

Myeloid angiogenic cells (MACs)

There has been recognition that the "EPC" as defined by Asahara and colleagues [3], and the many subsequent derivative methods (reviewed in [51, 64]), did not identify cells that could directly form vessels in vivo. Rather, that these cells were recruited to sites of vascular injury to secrete a variety of growth factors, chemokines, cytokines, and metalloproteinases to stimulate the endogenous vascular endothelial cells to repair the vascular damage was clarified by numerous key papers [65–74]. In brief, these papers clarified that bone-marrow-derived circulating myeloid lineage cells possess the capacity to upregulate expression of and/or access platelet microparticle-derived proteins upon in vitro culture that cause the cells to display morphologic and phenotypic features of endothelial-like cells. However, analysis of the cells demonstrates that these MACs possess a transcriptome, proteome, and ultrastructural features of monocyte-macrophages and do not form vascular structures in vitro or in vivo. It is clear that MACs promote vascular repair and regeneration at injury sites through the paracrine release of numerous biologically active molecules. Thus, MACs play important roles in participating in vascular endothelial repair and regeneration, but they do not transdifferentiate into cells of the endothelial lineage as originally reported [3]. It is this fact that should illuminate the importance of retiring the term "EPC" to refer to marrow-derived cells that circulate and adhere to sites of vascular damage when in fact these cells are MACs.

Definitions, lineage relationships, phenotypes, and functions

MACs display a cellular morphology typical of a circulating monocyte. All monocytes are derivatives of hematopoietic stem cells that reside in the bone marrow and are released into the circulation in a regulated fashion. The circulating monocytes express a variety of antigens including CD45, CD14, CD31, Tie2, CXCR4, CD11b, and CD115 [5, 75]. Upon in vitro culture and accumulation in tissues in vivo, MACs mature into macrophages. Those culture conditions that contain serum and various angiogenic growth factors promote the MAC to upregulate cell surface proteins that are more typically displayed by endothelial cells, and the MAC may even alter their phenotype to look like cultured endothelial cells [76, 77] but they do not become endothelial cells [68, 78, 79]. MACs play important roles in promoting vascular repair in numerous types of injury including limb ischemia [75] and promote vascularization of tumors [75, 80–83]. Both the circulating monocytes and tissue macrophages are recruited to areas of vascular damage [75] and to ischemic tissue environments where they support vessel repair and growth. The fact that MACs are attracted to sites of vascular injury, adhere to damaged or denuded vascular basement membrane, and secrete paracrine factors that promote resident vascular endothelial cell migration, proliferation, and maturation clearly identifies the important role of MACs in these processes. However, MACs are not "EPC" as they do not transdifferentiate into endothelial cells. Therefore, when using marrow-derived cells to study vascular repair and these cells display features of MAC, they should be called MAC and not "EPC." Are there any cells that can still be called "EPC?" Only those cells that become fully committed to the endothelial cell lineage and display the capacity to form new blood vessels at sites of injury fulfill the requirements to be called "EPC."

Endothelial colony-forming cells (ECFCs)

As noted earlier, there is consensus agreement that ECFCs display the capacity to directly form blood vessels in vivo upon implantation and as such, are the "true EPC" [5]. However, because the "EPC" nomenclature has been so confusing to investigators and clinicians, by consensus, the term ECFC has been chosen to refer to these vessel-forming cells [5, 84] as originally defined [85].

The knowledge that some endothelial cells circulate in the bloodstream of human subjects and numerous animal species traces back to publications in the late 1950s (reviewed in [63]). Techniques and approaches to quantitatively document differences in proliferative potential of human blood outgrowth endothelial cells [86] did not appear until the present century and were soon followed by reports for a hierarchy of ECFC circulating in human umbilical cord blood and adult peripheral blood [85]. As the volume of work supporting the role of ECFC in vascular repair and regeneration continues to grow, enthusiasm continues to swell for the translation of ECFC as a therapy to treat human cardiovascular diseases including peripheral arterial disease, stroke, and various states of ischemia and hypoxia [60, 61, 87, 88].

Definitions, lineage relationships, phenotypes, and functions

The term ECFC emerged from studies to define the proliferative hierarchy visually apparent in growing colonies that emerged from individual clones of circulating endothelial cells present in umbilical cord blood [85, 89]. The ECFC could be observed to display significant differences in number of progeny over a two-week in vitro culture; the ECFC clones with greatest proliferative potential gave rise to > 10,000 progeny, whereas some plated ECFC only divided 1–5 times [89] (Fig. 9.2). While few differences in cell surface expression of antigens between the low and high proliferative clones have been reported [89–91], differences in telomerase activity are evident with highest level of abundance in high proliferative ECFC [85, 89] and loss of high proliferative clones in patients with peripheral arterial disease with critical limb ischemia have been reported [92]. ECFCs derived from umbilical cord blood and adult peripheral blood display robust in vivo vessel formation in a variety of preclinical models of human ischemic and hypoxic disorders (Fig. 9.3) [87, 93].

The origin of circulating ECFC has remained elusive. Some evidence suggests that they are derived from transplantable whole bone marrow (not from hematopoietic but potentially from vascular elements) [86], while clear evidence for the presence of ECFC has been shown from resident vascular endothelium [89]. In fact, several reports have identified ECFC to be comprised of resident vascular endothelial stem and progenitor cells [94–99]. This topic has been reviewed recently [100–102]. Moreover, evidence is accumulating that infusion or injection of ECFC may serve as a potential cell therapeutic approach for numerous human disorders [87, 93]. Advances in single cell "omics" analysis may permit identification of resident vascular ECFC displaying tissue-specific properties [103–107] and thus allow tools to unlock mechanisms for organ and tissue-specific vascular dysfunction in aging, disease responses, and cardiovascular disorders [108–110].

Evidence for MAC and ECFC in acute and chronic kidney disease

Myeloid angiogenic cells in kidney injury and repair

Initial studies on the potential role of EPCs in kidney repair were accompanied by inconsistent criteria for definition as well as varied approaches in analysis. For example, CD34, a surface marker of mobilized HSC, is thought to be activated on EPCs that we now term MAC. The utilization of CD34 immunostaining may have been applied to evaluate EPCs in models of kidney injury [111]; however, since CD34 may also be expressed on endothelial cells, CD34 expression itself does not provide convincing discrimination between MAC and resident endothelium. Other approaches have utilized FACS, which combines CD34 expression with other markers such as Flk1 to demonstrate infiltration of "EPCs" likely corresponding to MAC. For example, selective vascular injury by injection of concanavalin A A into the renal artery was shown to induce mobilization of CD34 +/Flk1 + in the circulation within 3 h and their subsequent homing into the kidney was established within 3–5 days [112], suggesting activation of MAC in response to kidney endothelial injury. However, because of the overlap of surface marker expression between MAC and ECFC, data from studies utilizing FACS without discrimination of hematopoietic markers such as CD45 should be interpreted carefully.

Another approach to evaluate trafficking has been to utilize bone marrow transplant chimeric animals. Bone-marrow cells derived from the donor and expressing endothelial cell markers likely to correspond to MAC.

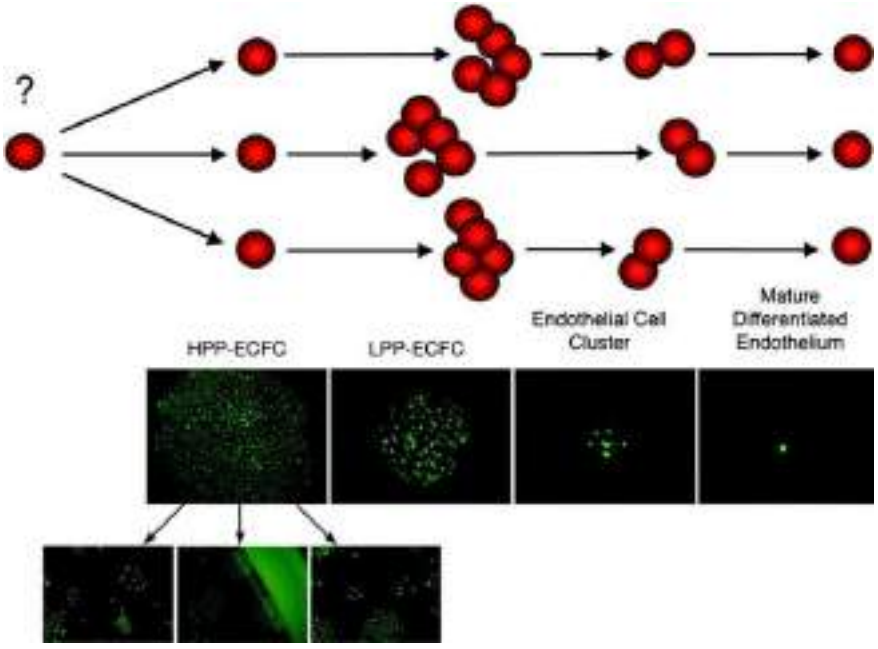

FIG. 9.2 Model of an endothelial progenitor cell hierarchy based on the proliferative and clonogenic potential of discrete populations of progenitor cells. **HPP**-ECFCs are large colonies form secondary and tertiary colonies on replating. HPP-ECFCs give rise to all subsequent stages of endothelial progenitors in addition to replating into secondary HPP-ECFCs. LPP-ECFCs form colonies that contain more than 50 cells but do not form secondary colonies or LPP-ECFCs on replating. Endothelial cell clusters can arise from a single cell but contain fewer than 50 cells that are typically larger than the smaller cells found in HPP-ECFC and LPP-ECFC colonies. Mature, terminally differentiated endothelial cells do not divide. *Reprinted with permission from Ingram DA, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood, 2004;104(9):2752–60.*

Several studies have demonstrated that these cells infiltrate into kidney or the renal vasculature in a variety of injury models such as ischemia/reperfusion, Adriamycin, or anti Thy1.1 antibody exposure [113–116]. The basis for MAC homing to injured kidney is multifold. Evidence provided by studies using renal artery stenosis model of microvascular damage in pigs or in rat models of ischemia reperfusion suggests that the damaged kidney expresses homing signals such as SDF-1 and angiopoietin-1 [117]. Blocking of SDF-1 activity dramatically attenuated homing of BM-derived cells to the kidney following ischemic injury [118]. Interestingly, increased circulating levels of CD34/VEGR2 + cells or CD133/VEGFR2 cells were found in patients following renal transplant and these correlated with plasma SDF-1 [119]. The homing activity of SDF-1 has been suggested to occur due to the upregulation of *E*-selectin-mediated adhesion [120].

The localization of these cells in the vasculature led initially to the idea that these circulating progenitor cells differentiate into vascular cells to repair the blood vessels. However, as stated earlier, there is no evidence that such cells integrate stably into the renal vasculature or form vessels in vivo. The data from kidney injury models are consistent with these observations. For example, Li et al. using a model of Adriamycin-induced injury showed the peak expression of infiltrating MAC occurred within 14 days of injury and declined within 28 days [121]. Sradnick et al. applied bone marrow (BM) transplanted td-tomato-labeled cells and investigated the trafficking of these cells following endothelial injury with ischemia reperfusion (I/R) or concavalin A. The results confirmed that BM-derived tdTomato + cells do not contribute to vasculature. Rather, BM-derived cells were typically inflammatory [122]. Similar conceptual approaches have been applied to study MAC in the setting of human transplant. Using a fluorescence in situ hybridization approach to distinguish host versus donor, Kwon et al. demonstrated an influx of CD34 + cells in kidney and a decrease in circulating CD34 + within 1 h of transplant. As many as 17% of cells located in or near the peritubular capillaries were derived from the recipient (i.e., from circulation). However, after 35 or 73 days following transplant, no recipient cells were found in the peritubular vasculature [123]. In contrast, a significant percentage of tubular cells were found to be derived from the recipient. Thus, these data are consistent with the view that MAC may home to sites of vascular injury, but do not substitute as endothelial progenitors.

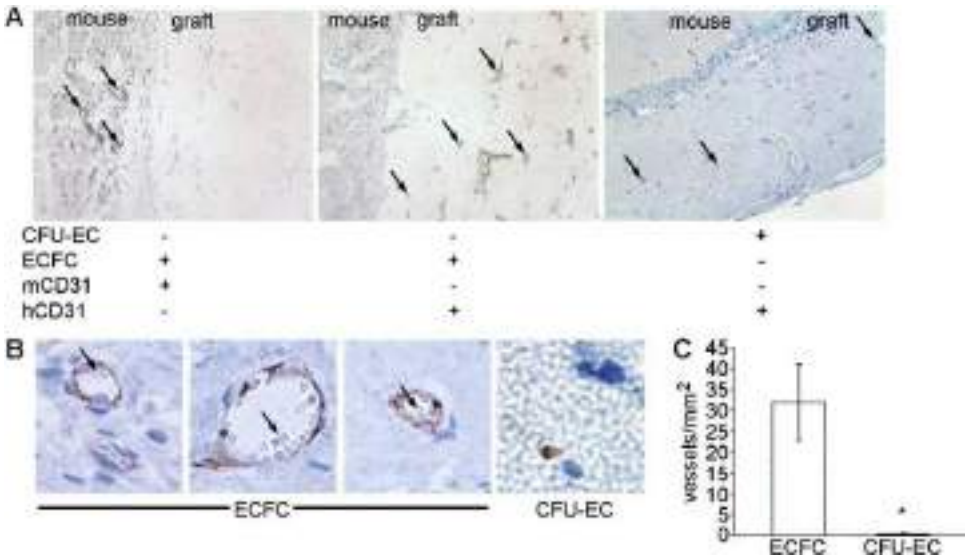

FIG. 9.3 Transplantation of CFU-ECs and ECFCs into NOD/SCID mice. (A) Photomicrographs (original magnification, × 20) of cellularized grafts and surrounding murine tissue 28 days after implantation into NOD/SCID mice. Left and middle panels show consecutive sections of the same ECFC graft stained with antimurine CD31 (mCD31) and antihuman CD31 (hCD31) to identify either murine or human blood vessels, respectively. mCD31 (left) does not cross-react with human endothelial cells within the graft and hCD31 (middle) does not cross-react with murine endothelial cells in the vessels outside the graft. Murine vessels were never identified in the cellularized graft ($n = 18$). Right panel shows a MAC comprised colony-forming unit "endothelial cell" (CFU-EC) graft stained with antihuman CD31. Arrows indicate positive antigen staining. Results represent 9 other ECFC grafts and 2 other CFU-EC grafts. (B) Photomicrographs (original magnification, × 100) of ECFC and CFU-EC (far right) cellularized grafts stained with antihuman CD31 28 days after implantation. Vessels and capillaries in ECFC grafts are perfused with murine red blood cells (arrows) indicating anastomoses with murine blood vessels. CFU-EC grafts fail to form vessels or capillaries. Results represent 9 other ECFC grafts and 2 other CFU-EC grafts. (C) Quantitation of capillary density within ECFC (□) and CFU-EC (■) cellularized grafts 28 days after implantation. Results represent the average number of capillaries containing murine red blood cells/mm^2 of graft tissue ± SEM of 10 ECFC and 3 CFU-EC grafts. *$P < 0.05$ by Student unpaired t test. *Reprinted with permission from Yoder MC, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood, 2007;109(5):1801–9.*

Endothelial colony-forming cells in kidney

Since ECFCs represent the true endothelial progenitors, it is important to investigate the potential role resident renal ECFCs and their potential to participate in renal repair. However, as yet, little work has been directed to this goal. Resident renal endothelial cells are characterized typically by slow proliferation rates. Our group evaluated doubling time of kidney vascular endothelial cells and showed these to divide far slower than EC derived from pulmonary artery or lung microvasculature [124]. In addition, rat kidney EC lacked the ability to form branching structures without support and did not proliferate in response to VEGF. Single cell colony-forming assays indicated that the majority of kidney EC remained as single cells or small clusters indicative of low proliferative potential ECFC [124]. To date, no other studies have attempted to evaluate kidney ECFC potential. Muczynski et al. isolated clones from human kidneys with the potential to form confluent monolayers [125]. However, since the clones were selected for growth, it is unclear what percentage of resident vascular cells have high proliferative capacity [125].

The low proliferation of cells derived from the resident kidney vasculature has been previously demonstrated by others. The inability to maintain primary cultures of rodent glomerular or peritubular capillary endothelial cells has been known for several decades and has only been alleviated by immortalization strategies [126]. Ligresti et al. demonstrated that human renal microvascular endothelial cells (RMECs) manifested slower growth, impaired sprouting activity, and lacked responsiveness to VEGF relative to HUVEC cells. These investigators also demonstrated downreguation of RGS5, MMP1 and an upregulation of DLL4, consistent with a lack of angiogenic potential [127]. RNAseq analysis from the same group compared mouse kidney EC gene expression with aortic and lung EC and demonstrated substantial differences in their molecular signatures. Kidney EC lacked expression of genes associated with VEGF signaling and expressed higher levels of PTEN and the transcription factor FoxO1 [128]. Because FoxO1 interacts with chromatin on VEGF-regulated genes, the investigators subsequently demonstrated that inhibition of FoxO1 enhanced kidney EC angiogenesis and also permitted better responsiveness to VEGF. Interestingly, inhibition of FoxO1 expression was associated with better vascular regeneration in response to renal ischemia reperfusion

injury [128]. These molecular signatures suggest that kidney-derived ECs intrinsically lack regenerative growth capabilities and angiogenic potential, which may be implicated in the susceptibility of renal vascular injury.

We suggest that the overall low rate of proliferation of these cells reflects upon the nature of resident ECFC activity tilted to a population with inherently low colony-forming activity in the kidney. Unfortunately, given the lack of a specific markers for ECFC at the current time, the basis for low endothelial proliferative potential in the kidney and the role of ECFC in growth and response to injury remain to be thoroughly addressed. We anticipate that the identification of better markers for ECFC will ultimately enable the study of vascular repair in the kidney with a focus on the role of discrete progenitor populations.

Hypothesis: Low ECFC activity and impaired MAC activity contribute to disease progression

We propose that inefficient vascular repair responses fundamentally underlie vascular rarefaction and predispose to CKD development. Few studies have described a prominent endothelial cell proliferation response following kidney injury [31, 129]. Several lines of evidence suggest that renal injury is associated with an activation of several anti-angiogenic factors, including angiostatin, endostatin, s-Flt1, ADAMTS-1, and TGF-beta [1, 25, 130–132]. Vascular injury or impaired regeneration may result from a loss in trophic support [25]. In addition, a reduction in the kidney expression of VEGF-A, a critical factor involved homeostatic support for existing blood vessels, is transiently reduced following I/R [132, 133] or is chronically reduced in CKD models with capillary rarefaction [25, 134]. The source of homeostatic VEGF expression is typically considered to be derived from tubules, podocytes, and/or pericytes. In the setting of disease, pericytes change their gene expression profile to produce anti-angiogenic factors such as ADAMTS-1 and lose their ability to stabilize endothelial branch formation using in vitro assays [135].

A second factor leading to inefficient repair may result from impaired MAC activity. MACs stimulate endothelial cell proliferation and migration and have been shown to induce angiogenic branch formation in vitro and in vivo [129, 136–138]. This activity may be reflective of their homeostatic function to maintain vascular integrity. The model proposed by Ingram, Yoder, and others suggests that infiltration of MAC influences the local milieu to promote vascular repair by stimulating local ECFC proliferation and branching for repair or angiogenesis [67, 68, 139, 140]. An impairment in the number or activity of MAC would be hypothesized to compromise vascular stability over time.

Goligorsky articulated the concept of "EPC incompetence." This concept was prompted in part by studies demonstrating that the number of circulating MAC or the activity of MAC is impaired in patients with cardiovascular risk factors [141–143]. Circulating MACs are also reduced in patients with CKD, and this may be a direct result of uremic environment [144–146]. Data from animal models are consistent with the concept that MAC activity influences renal vascular repair. For example, in obese Zucker rats with metabolic syndrome or in Zucker fatty diabetic rats, circulating MACs are reduced relative to lean Zucker controls. Moreover, BM-derived MACs displayed less differentiation and in vivo angiogenic activity relative to MACs from control rats [147]. In addition, Zhu et al. demonstrated that the initial injury phase in swine model following induction of renal artery stenosis increased the activity of circulating MAC. Under chronic stenosis conditions associated with vascular loss, fibrosis, and hypertension, endogenous MAC activity is significantly attenuated while the administration of exogenous MAC improved vascular structure and prevented renal fibrosis [148].

We propose that a final aspect of inefficient repair is due to the nature of resident ECFC [129]. While endogenous MACs are mobilized to injured kidney and hypothesized to contribute to vascular repair, such activity seems largely ineffective since vascular rarefaction remains the predominant feature of all models leading to renal fibrosis [25]. It is reasonable to suggest that the proportion of high proliferative potential ECFC residing within a given vascular bed is reflective of its vascular regenerative potential. Therefore, we suggest that an additional aspect of inefficient vascular repair is due to low intrinsic activity of resident tissue ECFC.

Several studies provided evidence that exogenous angiogenic factor support by either VEGF-121 or Ang1 could mitigate renal capillary loss in disease models [31, 133, 149–151]. It is unclear whether angiogenic support mediates protection by stimulating of proliferation resident cells or by injury prevention. In vivo, endothelial proliferation responses in injury or disease models are not well characterized and subject to limitations in co-labeling techniques. Some studies have described capillary sprouting and endothelial proliferation of glomerular endothelial cells in response to immune complex formation or diabetes, and few reports have identified proliferating EC in peritubular capillaries [152–156]. In one study, Hohenstein et al. identified proliferating peritubular capillary EC by co-staining PCNA with the mouse endothelial cell antigen MECA-32 following vascular injury with concavalin A, and this proliferation was enhanced by treatment with erythropoietin [112]. In contrast, following repetitive BrdU injection after renal I/R, evidence of BrdU + EC after 7 days was minimal. Moreover, VEGF-121, which preserved vascular density, did not influence endothelial BrdU incorporation. Rather, VEGF121 attenuated EndoMT [31].

It is reasonable to suggest that the degree of cell proliferation in kidney is insufficient to maintain vascular homeostasis, since loss of vessel density is typically considered permanent. VEGF-121, which attenuates vascular loss immediately following I/R, has no effect on restoring vascular density if the administration is delayed for 3 weeks [149], suggesting a lack of VEGF-induced angiogenic repair after rarefaction is established. However, some studies have demonstrated that loss of vascular density is potentially reversible. Most notably, studies by Chade and colleagues using the swine model of renal artery stenosis have shown that administration of either VEGF or MAC by intra renal artery injection 6 weeks following stenosis could reverse microvascular damage and attenuate CKD progression [157, 158]. Thus, at least within the swine, there is potential for an endogenous vascular repair response after established vascular injury. Whether these different results are due to species differences in renal EC proliferative potential or due to differences in models is not yet known.

Potential therapeutic benefit of PAC or ECFC

Based on impaired PAC or ECFC status in the kidney, the potential use of EPCs to promote vascular repair in kidney remains an attractive option. Repletion of either MAC or ECFC could be hypothesized to promote resistance to endothelial damage or promote vascular repair. Table 9.1 summarizes several studies in which exogenous administration of MAC, primarily early outgrowth cells, provides protective effects against renal injury. The diversity of different models and species represented in the table suggests that MAC may represent a useful therapeutic option in protecting or potentially reversing vascular injury. Increasingly, investigators have sought strategies to improve the activity of MAC by harvesting or treating cells under certain conditions to enhance potential beneficial effects [178]. For example, MACs pretreated in vitro with melatonin, angiopoietin-1, or BMP-7 enhance the protective capacity of MAC to protect mice from ischemic AKI [162].

The protective effect does not appear to require homing of the cells to the site of injury. Golle et al. showed that BM-MAC-derived cells restored vascular density and decreased the extent of fibrosis in a rat model of 5/6 nephrectomy, while engraftment and differentiation of exogenous MAC could not be detected. Experiments using conditioned media indicate that protective factors are secreted from these cells [168].

The protective effects mediated by bone-marrow-derived MAC are more than likely the result of multiple mechanisms. Since the cells do not differentiate into endothelium, it is proposed that they provide an enriched paracrine environment suitable for angiogenic repair [74, 179–181]. Studies from Goligorsky's laboratory indicate that treatment of rats with cells likely corresponding to MAC improves early alterations in renal hemodynamics in response to ischemic injury, while other studies suggest that MACs have anti-inflammatory properties [159, 178, 182]. Patschan et al. demonstrated that MACs not only provide resistance to acute tubular injury but also provide protection from peritubular capillary rarefaction following I/R and that MACs inhibit EndoMT [160, 161].

The pro-angiogenic effect of BM-derived PAC is likely, in part, due to paracrine release of factors such as VEGF, HGF, or G-CSF in the local environment [129, 136–138]. Studies based on mesenchymal stem cells or adipose-derived stem cells have described biological activity in the microvesicles secreted from these cells [183–185]. In some cases, "horizontal transfer" of miRNA by the vesicles to the target cell is thought to alter transcriptional control. This type of control likely also exists for BM-derived MAC. Deregibus et al. isolated MAC from circulating blood of healthy volunteers and isolated microvesicles which incorporated into human EC in culture and supported angiogenesis in vivo in SCID mice [186]. Microvesicles derived from bone-marrow-derived MAC were recently shown to be effective in reducing fibrosis and preventing myofibroblast transition in a mouse model of UUO [169]. However, there have been relatively few studies that have investigated MAC-derived microvesicles in renal disease models.

ECFC-based approaches have also been pursued with a goal of promoting recovery from renal injury (Table 9.1). Initial studies using transplantation of HUVEC, which are enriched in HPP ECFC, were shown to alleviate microvascular dysfunction in the setting of renal I/R injury [172]. Our group investigated the effect of rat lung-derived EC with high proliferative potential on AKI and demonstrated that they had a powerful protective effect against ischemia reperfusion injury, while pulmonary artery derived cells with low proliferative potential had no protective effect [21]. Similarly, studies from Burger et al. and Pang et al. demonstrated human ECFC from cord blood or EC from human renal artery had protective effects in SCID mice by reducing the degree of tubular injury and vascular rarefaction in response to I/R [170, 175].

Since ECFCs are endothelial cells, it was expected that these cells may engraft and participate directly in vascular repair. However, these studies all reported either no engraftment or minimal engraftment of the injected cells into the kidney [21, 170]. The reason for lack of engraftment of these cells is not known, but these reports suggest that the protective activity can be achieved by factors released from cells, similar to the effects described for MAC. In support of this notion, conditioned medium from human cord blood derived ECFC protected against the acute effects of I/R.

TABLE 9.1 Evidence of renal protection by MAC or ECFC in animal models of disease.

Author, reference, year	Cell type	Biological source	Model/biological effect
Patschan et al. [159], 2006	MAC	Murine spleen and kidney	Unilateral I/R in FVB/NJ and Tie-2-GFP mice; reduced plasma creatinine by ~ 50% post I/R; no evidence of homing
Patschan et al. [160], 2015 Patschan et al. [161], 2016	MAC	Murine peripheral blood and spleen	Bilateral I/R in male C57BL/6N mice, reduced plasma creatinine, increased endothelial autophagy, decreased EndoMT, and attenuated capillary rarefaction
Patschan et al. [162], 2012 Patschan et al. [163], 2013 Patschan et al. [164], 2013	MAC	Murine peripheral blood and spleen	In mouse I/R, pretreatment of cells with melatonin, angiopoietin-1, and BMP-5 enhances protective response, with minimal homing
Chen et al. [165]	MAC	Murine bone marrow	Labeled cells home to kidney and improve function in response to I/R injury
Rookmaker [166]	MAC	Murine bone marrow	Rat model of Thy1.1 antibody. Increased expression of RECA positive cells after injury
Sangidorj et al. [167]	MAC	Murine bone marrow	Protection against CKD progressive loss of function and injury in mouse model of 5/6 Nx
Golle et al. [168], 2017	MAC and MAC-derived exosomes	Rat bone marrow	Protection against loss of GFR and fibrosis in a rat model of 5/6 nephrectomy
Yang et al. [169], 2019	MAC and MAC-derived vesicles	Mouse bone marrow	Vascular protection in a mouse model of unilateral ureteral obstruction (UUO)
Chade et al. [117]	MAC	Swine peripheral blood	In model of renal artery stenosis, injected cells home near the vasculature. Improve vascular injury and fibrosis
Burger et al. [170], 2015	ECFC and ECFC exosomes	Human umbilical cord blood	Reduced plasma creatinine by ~ 40%. Improved tubular morphology without evidence of homing following I/R in NOD-SCID mice
Collett et al. [21], 2017	ECFC and ECFC-conditioned medium	Rat pulmonary microvascular EC and human ECFC	Bilateral I/R in male Sprague Dawley rats; reduced plasma creatinine by ~ 50% post-I/R; improved tubular morphology; prevented loss of medullary perfusion; no evidence of homing
Viñas et al. [171], 2016	ECFC and derived exosomes	Human umbilical cord blood ECFC	Bilateral I/R in male FVB mice; reduced plasma creatinine by ~ 90% post-I/R; improved tubular morphology
Brodsky et al. [172], 2001	EC	Human umbilical cord	Unilateral I/R in male athymic rats; reduced plasma creatinine by ~ 50% post-I/R; maintained microvascular perfusion; minimal evidence of homing
Cantaluppi et al. [173], 2012	ECFC and derived exosomes	Human peripheral blood	Unilateral I/R in male Wistar rats; reduced plasma creatinine by ~ 80% post-I/R; improved tubular morphology; prevented capillary rarefaction
Liang et al. [174], 2015	EC	Human umbilical cord (Whartoń s jelly)	Unilateral I/R in male C57BL/6 mice; reduced plasma creatinine by ~ 70% post-I/R; improved tubular morphology and capillary rarefaction; minimal homing
Pang et al. [175], 2017	EC or ECFC	Human renal artery	Bilateral I/R and reduced renal mass in NOD-SCID mice. Reduced plasma creatinine by ~ 50% post-I/R; prevented capillary rarefaction; minimal homing near peritubular capillaries up to 10 days
Patschan et al. [176], 2017	Indeterminate	Murine peripheral blood and spleen	Bilateral I/R in male C57BL/6 mice; reduced plasma creatinine by ~ 10% at 1 week did not prevent capillary rarefaction
Zullo et al. [177], 2015	Indeterminate	Murine embryonic EPC line cultured with MSCs	Improved renal function, medullary RBF, and increased M1 to M2 macrophage polarization in LPS-induced endoxemia in C57BL6 mice

Adapted and modified from Basile DP, Collett JA, Yoder MC. Endothelial colony-forming cells and pro-angiogenic cells: clarifying definitions and their potential role in mitigating acute kidney injury. Acta Physiol, 2018;222(2):12914.

This response was associated with preservation in medullary blood flow within 2 h of reperfusion, inhibition of endothelial ICAM1 expression, and attenuation of T-cell infiltration [21].

In a recent study from Cantiluppi et al., extracellular vesicles derived from ECFC were shown to localize within peritubular capillaries and tubular cells [173]. These microvesicles protected against the development of renal tubular damage as well as the development of renal capillary rarefaction and subsequent renal fibrosis following I/R. Treatment of microvesicles with Dicer or antimir 126 or 296 blocked the protective effects consistent with the concept of "horizontal transfer" of miRNA as the basis of protection [173, 187]. Studies from Vinas et al. demonstrated that human cord blood-derived ECFC exosomes contain high levels of microRNA 486-5p and antagomir treatment to block miR486-5p attenuated the protective effect of these exosomes in response to ischemia [171]. The exosomes manifest effects in target endothelial cells, in part, by decreasing endothelial expression of PTEN and increasing Akt [171].

Concluding remarks, future directions

Much has been learned regarding the nature of multiple cell types with the potential to improve vascular repair. Since the original identification of myeloid cells with angiogenic potential, the study of endothelial progenitors has matured. In this chapter, we have described recent consensus updates in terminology regarding the definitions of "EPCs" as either myeloid-derived angiogenic cells (MACs) or endothelial colony-forming cells (ECFCs). Evidence of the activity of these cells in renal vascular repair has been updated in preclinical models.

A number of important issues lie on the horizon. First, will any progress be made regarding translating these results into the clinical setting? The use of MAC in phase II clinical trials has been described. In one recent study, autologous peripheral blood-derived CD34 + cells in patients with stage III or IV CKD were investigated and demonstrated safety and improved renal outcomes after 12 months [188]. In contrast to the relative ease of isolating and processing MAC for clinical studies, the growth and expansion of ECFC for potential therapy in patients require significantly greater efforts [189–191].

Second, given the new emergence of consensus on definitions, it is critical that all new studies undertake steps to characterize the nature of the cells used in preclinical studies. Recently, Liao et al. reviewed a large number of studies investigating cardiovascular preclinical models that purported to use ECFC intervention and found that only a small proportion of these studies utilized cells that fulfilled "Medina" criteria [192]. With increased awareness, we anticipate greater attention to the phenotypes of cells used in future studies.

A final issue relates to the lack of homing of ECFC in preclinical models. While paracrine effects of ECFCs on kidney function are exciting, the long-term goal of using this strategy to help promote revascularization is not practical unless engraftment is achieved. Alternatively, we predict that a better understanding of resident ECFC represents a better approach to promote revascularization. Identification of a specific markers which help define the activity and fate of ECFC in local tissues will help to push strategies to improve ECFC function and facilitate better repair response. With the increased attention of omics technologies and increased awareness of discrete "EPC" populations, we look forward to significant advances in this area to enable better strategies to facilitate renal vascularization and improve kidney function.

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CHAPTER

10

Stem cells in regenerative processes: Induced pluripotent stem cells

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Introduction

With few curative treatments for chronic kidney disease (CKD), the only effective treatment option for the increasing number of patients worldwide with end-stage renal disease (ESRD) is dialysis therapy, which causes medical and medicoeconomic problems [1]. Renal transplantation has proven a successful therapy for most ESRD patients, significantly improving the patient's quality of life (QOL) and prolonging survival while also being cost-effective. However, the annual increase in the number of ESRD patients who need a renal transplant and the widening gap between the demand for and supply of donor kidneys has led to a progressive shortage of donor organs. This shortage has become a serious issue and is even worsened by the problem of limited graft survival due to immune rejection [1].

Among strategies to overcome these problems is regenerative medicine using stem cells. Stem cells are largely divided into two categories: organ-specific (or somatic) stem cells and pluripotent stem cells (PSCs). In contrast to organ-specific stem cells that generally have a limited potential for growth and differentiation, PSCs, such as embryonic stem cells (ESCs) [2–4] and induced pluripotent stem cells (iPSCs) [5–7], have a virtually unlimited replicative capacity on culture dishes and are theoretically able to give rise to any cell type in the body. In addition, these stem cells have increasingly been used as a model system for understanding developmental mechanisms. Furthermore, in vitro culture and differentiation protocols offer unique opportunities for disease modeling, drug discovery, toxicology, and cell replacement therapy [8]. Because of the complexity of the kidney developmental process and structures, progress in the directed differentiation of kidney lineages from PSCs had been slow, but techniques have advanced greatly in the last decade.

Among therapeutic approaches, using human ESCs face two major problems. One is the ethical issue concerning the use of human fertilized eggs and the other is the immune rejection of an ESC-derived cell or tissue transplantation product due to histocompatibility antigenic differences between the ESCs and patients. These problems have been overcome by breakthrough research by Takahashi and Yamanaka [5, 6]. They identified four factors normally found in ESCs, OCT4, SOX2, KLF4, and c-MYC, which are sufficient to reprogram both mouse and human somatic cells to a state that closely resemble mouse and human ESCs and named the resultant cells iPSCs [5, 6]. Around the same time, Yu et al. generated human iPSCs with a different combination of factors that included OCT4, SOX2, NANOG, and LIN28 [7]. Since iPSCs can be generated from somatic cells of the patients themselves, clinical approaches using iPSCs are not associated with the aforementioned two problems.

This chapter first summarizes the recent advances in iPSC technology, developmental biology of the kidney, and the generation of kidney lineage cells and tissues from PSCs. Then, the current status and future perspective of the applications of iPSCs in the field of regenerative nephrology is described, such as cell therapy, kidney reconstruction, and disease modeling.

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iPSCs

Since the first report of iPSCs, the technique has been rapidly confirmed and improved. This technology has since given birth to an entire research field that has progressed at a phenomenal pace. The original method to generate iPSCs used genome-integrating viral vectors, retroviral or lentiviral vectors [5–7], which all risk carcinogenesis. To overcome this issue, several alternative iPSC production methods have been reported [8]. Although the iPSCs generated with these second-generation methods are similar to those generated with the original methods, the iPSC generation has lower efficiency and reproducibility. Okita et al. established a novel generation method by using episomal vectors encoding OCT4, SOX2, KLF4, L-MYC, LIN28, and a shRNA for TP53 [9, 10]. This system eliminates the integration of reprogramming factors into the genome of the somatic cells and derives iPSCs from any cell type with similar efficiency as the original methods.

One of the advantages of iPSCs is that they allow for autologous cell therapy using the patients' own iPSCs, thus avoiding the use of immunosuppressants. However, cell therapies using autologous iPSCs require much time and cost. It takes approximately 1 year to derive the iPSCs, confirm their safety and quality, and differentiate them to the desired cell types for the transplantation [8]. Allogenic iPSCs reduce both the time and cost. Accordingly, iPSC stocks are being made from healthy donor subjects whose three major human leukocyte antigen (HLA) loci, HLA-A, HLA-B, and HLA-DRB1, are homozygous [9]. HLA-matched allogenic transplantation is expected to lower the risk of immune rejection following the transplantation. At our institute, such homozygous HLA iPSC lines are generated with the episomal vector method, stored at clinical grade, and distributed to medical institutes where the cells are used for basic research and clinical applications [8].

The first clinical trial of a cell therapy using human iPSCs was in 2014 and transplanted autologous retinal pigment epithelial cells into a patient with age-related macular degeneration [11]. Thereafter, cell therapies using the earlier discussed allogenic HLA homozygous iPSC lines were performed for some intractable disorders including again age-related macular degeneration as well as Parkinsońs disease [12]. At the same time, therapeutic drug candidates have been identified by chemical screenings using iPSCs generated from patients with genetic disorders including fibrodysplasia ossificans progressiva (FOP) [13], amyotrophic lateral sclerosis (ALS) [14, 15], or Alzheimer's disease [16]. Clinical trials of the identified candidate compounds are currently under way.

Kidney development

The directed differentiation of iPSCs into kidney lineages has generally adopted the strategy of mimicking the developmental processes of the kidney (Fig. 10.1A). Vertebrates successively develop three kidneys: pronephros, mesonephros, and metanephros [17]. The three kidneys consist of a basic functional unit, the nephron, although the number of nephrons differs among the three. The three kidneys are derived from an early embryonic germ layer, intermediate mesoderm (IM), that is located between lateral and paraxial mesoderm. A lineage tracing experiment demonstrated that IM cells expressing *Odd-skipped related* (*Osr*)*-1*, the earliest marker for IM and an essential transcriptional factor for IM and kidney development, give rise to all components consisting of mammalian adult kidney metanephros [18]. Metanephros is formed by the reciprocal interaction between two tissues derived from IM, metanephric mesenchyme (MM) and ureteric bud (UB) [19]. The UB induces the MM to differentiate into the epithelia constituting nephrons and interstitium. Taguchi et al. closely analyzed the developmental process of mouse metanephros and revealed that the fertilized mouse egg first forms primitive streak on embryonic day (E)7.5, which then gives rise to two embryonic tissues, anterior IM on E8.5 and posterior IM on E9.5. The former forms the UB on E11.5 through the nephric (Wolffian) duct, while the latter forms the MM on E11.5 [20]. Thus, the cell fate of IM depends on the anterior and posterior domains.

By creating a novel culture system that uses the coculture of NIH-3T3 fibroblasts expressing a renal-epithelializing factor, *Wnt4*, Osafune et al. demonstrated previously that mouse MM contains multipotent progenitor cells that can give rise to several kinds of epithelial cells found in the adult nephron, including glomerular podocytes and epithelia of the proximal and distal renal tubules and of Henle's loop [21]. These progenitor cells are contained only in the cell population strongly expressing *Sall1*, a zinc finger transcriptional factor that is essential for kidney development. It was also shown that the progenitor cell population can reconstitute a three-dimensional (3D) kidney structure in vitro that contains glomeruli- and tubule-like components in an organ culture setting [21]. Later, the presence of the progenitor cells was also demonstrated in vivo by a lineage tracing experiment for *Six2*, which is also an essential transcriptional factor for formation of the kidney [22]. These progenitors are known as nephron progenitor cells (NPCs).

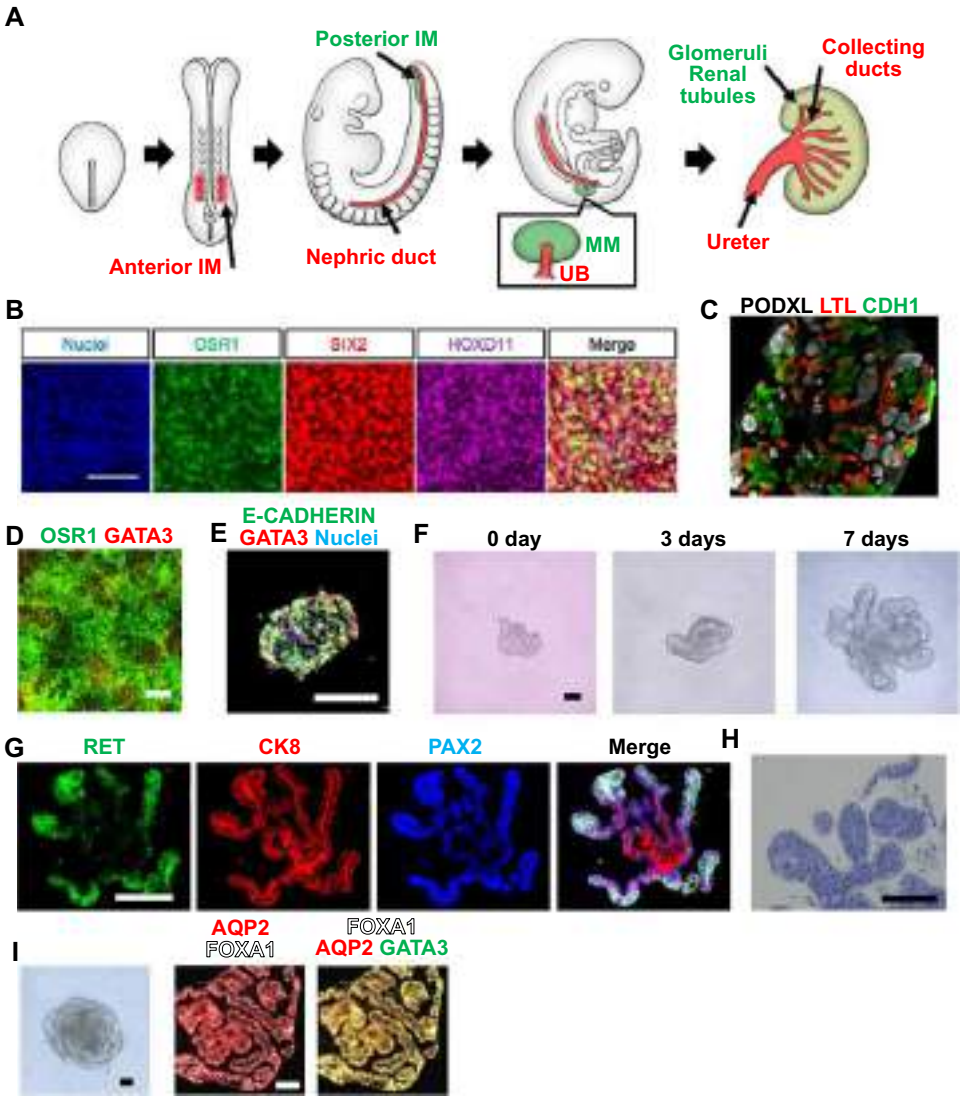

FIG. 10.1 Directed differentiation into kidney lineages. (A) A schematic showing mammalian kidney development. (B) Immunostaining for OSR1, SIX2, and HOXD11 in nephron progenitor cells (NPCs) differentiated from human iPSCs. (C) Triple immunostaining for PODOCALYXIN (PODXL; podocyte marker; *white*), *Lotus tetragonolobus* lectin (LTL; proximal tubule marker; *red*), and CADHERIN 1 (CDH1; distal tubule marker; *green*) in a nephron organoid formed from human iPSC-derived NPCs after 10 days of air-liquid interface culture. (D,E) Immunostaining of anterior intermediate mesoderm (IM) cells for OSR1 *(green)* and GATA3 (*red*; D) and a nephric duct cell aggregate for *E*-CADHERIN *(green)*, GATA3 *(red)*, and nuclei (*blue*; E). (F) Morphological change during the reconstruction of branching UB organoids for 7 days. (G) Immunostaining of a UB organoid for RET *(green)*, CK8 *(red)*, and PAX2 *(blue)*. (H) Toluidine blue staining of a UB organoid showing tubular lumens. (I) Bright-field *(left panel)* and immunostaining images *(middle and right panels)* of collecting duct-like tubular structures derived from a UB organoid for FOXA1 *(white)*, AQP2 *(red)*, and GATA3 *(green)*. Scale bars, 100 µm. *Modified from (B,C) Tsujimoto H, Kasahara T, Sueta S, Araoka T, Sakamoto S, Okada C, Mae SI, Nakajima T, Okamoto N, Taura D, Nasu M, Shimizu T, Ryosaka M, Li Z, Sone M, Ikeya M, Watanabe A, Osafune K. A modular differentiation system maps multiple human kidney lineage from pluripotent stem cells. Cell Rep 2020;31(1):107476; (D,E–I) Mae SI, Ryosaka M, Toyoda T, Matsuse K, Oshima Y, Tsujimoto H, Okumura S, Shibasaki A, Osafune K. Generation of branching ureteric bud tissues from human pluripotent stem cells. Biochem Biophys Res Commun 2018;495(1):954–61; Mae SI, Ryosaka M, Sakamoto S, Matsuse K, Nozaki A, Igami M, Kabai R, Watanabe A, Osafune K. Expansion of human iPSC-derived ureteric bud organoids with repeated branching potential. Cell Rep 2020;32(4):107963.*

NPCs differentiate into epithelia after Wnt stimulation, but a cell fate decision is required for further differentiation toward glomerular podocytes, proximal or distal renal tubules, or Henle's loop. Molecular mechanisms of the cell fate decision are largely unknown, though it was demonstrated that *Notch2* is required for the differentiation of proximal nephron structures, such as podocytes and proximal renal tubules, since *Notch2* deletion leads to the impaired formation of these proximal structures [23]. The MM may well contain at least two other precursor populations in addition to NPCs: vascular and stromal progenitor cells. Kobayashi et al. conducted a lineage tracing experiment for *Foxd1* gene and demonstrated that *Foxd1*-expressing cortical stroma represent a distinct multipotent

self-renewing progenitor population that gives rise to stromal tissues of the interstitium, mesangium and pericytes, although a small subset of Foxd1(+) cells contributes to *Six2*-expressing cells at the early stage of kidney outgrowth [24]. The UB is known to elaborate the lower urinary tract system from the collecting ducts through the renal pelvis and ureters to part of the urinary bladder [19]. Most of the mechanisms by which IM gives rise to kidney progenitors, the proximal-to-distal segmental patterning of nephrons from the glomeruli to distal tubules is formed, and the collecting duct system is elaborated from the UB are unknown. Elucidating these mechanisms will aid in understanding the commitment of multiple cell lineages within the kidney and reproduce these events in vitro from PSCs.

Directed differentiation into kidney lineages

In 1998, Thomson et al. showed the presence of glomerulus- and renal tubule-like structures in teratomas formed from human ESCs by transplanting them into immunodeficient mice, which suggested that kidney lineage cells can be generated from human PSCs [4]. Thereafter, by mimicking the developmental process of kidneys, directed differentiation methods that induce human PSCs into kidney lineages stepwise have been investigated. Song et al. for the first time generated glomerular podocyte-like cells from human iPSCs by treating them with activin A, bone morphogenetic protein (BMP)7, and retinoic acid (RA) [25]. Mae et al. derived the reporter human iPSC lines to monitor the expression of OSR1 (OSR1-GFP knockin human iPSC lines) and efficiently induced the cells into OSR1(+) IM cells at more than 90% differentiation efficiency using a combination treatment of activin A, BMP7, and a glycogen synthase kinase (GSK)3β inhibitor (CHIR99021), which activates the canonical Wnt/β-catenin signaling pathway [26]. These induced IM cells can further differentiate into adult renal cell types, such as glomerular podocytes and renal tubule epithelia, and form 3D renal tubule-like structures in vitro. Araoka et al. developed a compound-based differentiation protocol using a combination of CHIR99021 and two RA analogs, TTNPB or AM580, without any growth factors to induce nephrogenic IM cells from the OSR1-GFP knockin human iPSC lines [27]. Lam et al. also achieved a rapid and efficient generation of IM cells from human PSCs with 70%–80% efficiency after 3 days of their two-dimensional (2D) differentiation protocol, which further formed apically ciliated tubular structures expressing proximal tubule markers [28]. They also differentiated the IM cells into cells expressing the NPC markers SIX2, SALL1, and WT1.

Thereafter, several studies reported the selective induction of NPCs through posterior IM. Taguchi et al. differentiated embryoid bodies (EBs) of mouse ESCs or human iPSCs through epiblast, nascent mesoderm, and posterior nascent mesoderm into posterior IM and NPCs, though the induction efficiency of the NPCs was not high (around 40%) [20]. Notably, they were the first to generate nephron organoids containing both glomeruli and renal tubules from the induced NPCs. They later showed that the induced glomerular podocytes in the organoids exhibited apicobasal polarity with NEPHRIN proteins accumulated close to the basal domain and possessed primary processes that were connected with slit diaphragm-like structures [29]. An analysis of isolated human iPSC-derived podocytes showed the expression of well-conserved marker genes previously shown in mouse and human podocytes in vivo. Takasato et al. reported that a prolonged supplementation of CHIR99021 to human PSCs shifts the cell population from a more GATA3(+) anterior IM population to a more HOXD11(+) posterior IM and that the mixture of these two distinct IM populations spontaneously enhanced the formation of self-organized kidney organoids which contain multiple renal cell types, such as glomeruli, renal tubules, collecting ducts, and interstitial and vascular cells [30]. However, later studies with single-cell RNA sequencing (scRNA-seq) suggested that the induced GATA3(+)CDH1(+) ureteric epithelia included in the organoids could be distal renal tubules or connecting tubules derived from NPCs, because the organoids did not contain cells expressing the UB tip-specific markers RET and WNT11 [31, 32]. Morizane et al. also developed a simple 2D culture to efficiently generate NPCs from human PSCs at around 90% induction efficiency [33]. More recently, Tsujimoto et al. developed an efficient six-step differentiation protocol to induce multiple human iPSC lines through several stages of primitive streaks and posterior IM into NPCs at around 90% efficiency, more accurately reproducing the developmental process of NPCs and showing the developmental capability to form nephron organoids (Fig. 10.1B and C) [34].

Selectively controlling the differentiation of human NPCs to glomerular podocytes has been achieved [35]. By investigating the effects of various growth factors in cultures of mouse embryonic NPCs differentiating to podocytes, Yoshimura et al. successfully induced human iPSCs to selectively differentiate into podocytes with more than 90% purity using a phase-specific manipulation of Wnt and transforming growth factor (Tgf) β signaling. The induced podocytes exhibited global gene expression signatures comparable to those of adult human podocytes, podocyte morphologic features, including foot process and slit diaphragm-like structures, and functional responsiveness to drug-induced injury [35].

Regarding UB and collecting duct lineages, Xia et al. first reported the generation of UB progenitor IM cells from human iPSCs [36]. The IM cells differentiated and contributed to mouse UB tissues when cocultured with dissociated mouse metanephric cells in an organ culture setting. In later years, two groups developed directed differentiation methods to induce PSCs into UB-like tissues [37, 38]. Taguchi and Nishinakamura differentiated both mouse ESCs and human iPSCs into 3D UB-like tissues using EB formation through anterior IM and nephric ducts [37]. However, the induction efficiency of nephric duct epithelia was low at around 40%, and purification using flow cytometry and cell sorting with a monoclonal antibody against the cell surface markers CXCR4 and KIT was required for the subsequent differentiation into UB-like tissues. Mae et al. developed an efficient 2D monolayer differentiation method to induce human iPSCs into anterior IM cells and nephric duct epithelia at more than 70% efficiency (Fig. 10.1D and E) [38]. These cells proceeded to form UB-like tissue through simple mechanical pipetting to remove off-target cells. However, although the UB-like tissues generated with either method contained distinct tip and trunk domains, they showed limited branching morphogenesis. More recently, Mae et al. improved their UB differentiation method by culturing with low concentrations of Matrigel, which was reported to promote the formation of tubular lumen and apicobasal polarity in human PSC-derived inner ear organoids [39], and succeeded in generating UB organoids that exhibited apicobasal polarity, tubular lumen, and repeated branching potential (Fig. 10.1F–H) [40]. The separated tip regions from the organoids repeated the branching morphogenesis and reconstituted UB organoids again. This reconstitution process was repeated three to four times, and each tip region of the organoids repeated branching four to five times, indicating that each tip of the organoid maximally repeated branching 20 times like their counterparts in embryonic kidneys. Furthermore, the group succeeded in developing a differentiation method to further induce human iPSC-derived UB organoids to differentiate into collecting duct progenitor cells expressing AQP2, which was shown to be equivalent to their counterparts in gestational week (GW)7 embryonic kidneys based on the gene expression analysis (Fig. 10.1I) [40].

Expansion culture of kidney progenitors

Several groups have developed in vitro expansion culture methods for mouse and human embryonic NPCs in order to obtain a large amount of NPCs for both basic and clinical applications [41–43]. Brown et al. were the first to report the expansion culture of mouse embryonic NPCs by developing nephron progenitor expansion medium (NPEM), which recapitulates the niche signals for NPCs, by supplementing APEL medium used in monolayer culture settings with fibroblast growth factor (FGF)9, heparin, BMP4, BMP7, an ALK2/3 kinase inhibitor (LDN193189), CHIR99021, a Rho kinase inhibitor (Y-27632), insulin-like growth factor (IGF)1, IGF2, and Matrigel [41]. The method expanded CITED1(+) mouse embryonic NPCs up to one billion-fold while maintaining the developmental competence to form renal tubules in an air-liquid interface organ culture [41]. Tanigawa et al. developed an expansion culture condition for mouse embryonic NPCs using the combination of FGF9, TGFα, heparin, Y-27632, CHIR99021, leukemia inhibitory factor (LIF), a γ-secretase inhibitor (DAPT), and BMP7 with NPC aggregates plated on iMatrix-coated plates [42]. The study expanded E11.5 Six2(+) mouse NPCs more than 1800-fold after 19 days while maintaining their competence to form 3D nephron structures containing glomeruli and renal tubules when combined with embryonic spinal cord, which is a potent Wnt-expressing inducer of the mesenchyme-to-epithelial transition [42]. Based on the knowledge of NPC proliferation and by screening growth factors and chemical compounds using mouse NPCs derived from Six2-GFP mice, Li et al. established the nephron progenitor self-renewal (NPSR) medium, which consists of BMP7, FGF2, heparin, Y-27632, LIF, CHIR99021, an ALK4/5/7 kinase inhibitor (A83-01), and LDN193189 [43]. Using NPSR medium and 3D floating cell aggregation cultures, the group expanded Six2(+) mouse NPCs over 17 months with more than 110 passages. In addition, they succeeded in extracting NGFR(+)EPCAM(+) human NPCs from GW11 human fetal kidneys and maintaining the human NPCs for more than 7 months with more than 50 passages under NPSR medium. Both mouse and human fetal NPCs maintain the developmental potential to form nephron organoids in vitro upon exposure to a 2-day treatment with FGF2 and CHIR99021 after the expansion culture with NPSR medium. When the expanded NPCs were transplanted in vivo into the omentum of immunodeficient mice, the grafts functionally coupled to the host's circulatory system and produced urine-like metabolites via filtration. The long-term-cultured NPCs were also amenable to gene targeting using the CRISPR-Cas9 system to generate disease models in addition to cell therapy, drug screening, and toxicity testing [43].

Although the in vitro maintenance cultures for NPCs described earlier all use BMP7, the detailed mechanisms by which BMP7 acts in NPC expansion remain to be elucidated. Tsujimoto et al. unbiasedly screened chemical compounds for BMP7 substitutes and identified a JAK3 inhibitor, TCS21311, which can replace BMP7 in the expansion culture reported by Li et al. [43, 44]. Thus, the group revealed a novel inhibitory role of BMP7 in JAK3-STAT3

signaling for the NPC expansion culture. When added to the expansion culture, TCS21311 improved the expansion efficiency for both mouse embryonic and human iPSC-derived NPCs by around 30% [44].

Unlike mouse and human fetal NPCs, the in vitro expansion of human PSC-derived NPCs has not been achieved. Brown et al. were able to expand human ESC-derived NPCs differentiated with the induction method developed by Takasato et al. [30] with only two passages in NPEM medium while maintaining the developmental competency to form renal tubule-like structures [41]. Tanigawa et al. expanded the mouse ESC- and human iPSC-derived NPCs induced by their own induction method [20] for only 7 and 8 days, respectively, without passage using a combination of FGF9, TGFα, heparin, Y-27632, CHIR99021, LIF, DAPT, and BMP7, which was originally developed for mouse embryonic NPCs [42]. Later, the same group found that by examining and comparing various TGFβ-related ligands, activin A has superior effects to BMP7 on the maintenance efficiency of human iPSC-derived NPCs, expanding the human NPCs by only 5-fold per week over 2 weeks with one passage (up to 25-fold), while the cells retained a robust capacity for nephron formation both in vitro and in vivo [45]. This method was also applicable to multiple human iPSC lines. They also developed freezing methods for the single-cell suspension of human iPSC-derived NPCs, showing thawed human iPSC-derived NPCs were comparable with unfrozen NPCs in terms of the developmental potential to form nephron structures [45]. Li et al. expanded NPCs induced from a SIX2 reporter human iPSC line by the differentiation method developed by Taguchi et al. [20] for approximately 6 months by using NPSR medium [43]. However, the expansion method they used is only applicable to a small number of human iPSC lines. The development of robust expansion culture methods that can be broadly applied for multiple human PSC lines is required.

For the expansion of another kidney progenitor, UB cells, Mae et al. succeeded in expanding single cells dissociated from human iPSC-derived UB organoids to form colonies expressing two tip markers, RET and GATA3, by using a culture on soft hydrogel [40]. They also developed a monitoring method for tip cells by utilizing the capability of tip cells to uptake fluorescent dye-conjugated very low-density lipoprotein (VLDL) through VLDL receptors expressed on their cell surface and demonstrated that both tip and trunk cells expand to form RET(+)GATA3(+) tip colonies, indicating that trunk cells differentiate into tip cells as reported in mouse UB trunk cells [46]. These tip colonies derived from either tip or trunk cells reconstitute UB organoids, suggesting the successful expansion of UB cells. The group also developed freezing methods for nephric duct cells, which are the progenitor of UB cells [40].

Cell therapy

In addition to the reconstruction of kidney organs, human iPSC-derived kidney lineage cells have been examined for cell therapies [47,48]. Imberti et al. generated NPC-like kidney progenitors from human iPSCs by their original differentiation method and injected the progenitors through the tail vein of immunodeficient acute kidney injury (AKI) mouse models induced by cisplatin administration [47]. Some injected progenitors integrated into host mouse kidney parenchyma and differentiated into renal tubular cells. This therapy significantly suppressed the elevation of blood urea nitrogen (BUN) and the histological injury typical of AKI, such as tubular necrosis and cast formation, in host mice. Although the injected progenitors were reported to contribute to host renal tubules, the transplanted cells integrated in small parts of the recipient's renal tubules. It was therefore assumed that the therapeutic effects were not attributable to the integration of the injected kidney progenitors but to the paracrine effects by humoral factors secreted from the progenitors.

Toyohara et al. generated reporter human iPSC lines for OSR1 and SIX2 (OSR1-GFP/SIX2-tdTomato knockin human iPSC lines) and induced the cells into OSR1(+)SIX2(+) NPC-like kidney progenitors at 30%–40% efficiency by their original differentiation method [48]. They purified the OSR1(+)SIX2(+) progenitors by flow cytometry sorting and transplanted them into the renal subcapsular space of immunodeficient AKI mouse models induced by ischemia-reperfusion injury with unilateral nephrectomy. The cell therapy significantly suppressed the elevation of BUN and serum creatinine values of the host mice compared with saline injection or the transplantation of undifferentiated human iPSCs (Fig. 10.2A). In addition, the treatment significantly ameliorated the histological injury of AKI, including tubular necrosis, urinary cast, tubular dilatation, and loss of the tubular brush border, as well as interstitial fibrosis, which is a marker of the transition to chronic disease (Fig. 10.2B). The transplanted progenitors were not integrated into host kidney parenchyma, suggesting again that the main mechanism of the therapeutic benefits was paracrine effects by humoral factors secreted from the progenitors. Indeed, the study also showed that renal progenitors secreted multiple previously reported renal trophic factors, such as angiopoietin 1, vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF). In addition, a comparison of the therapeutic effects of purified OSR1(+)SIX2(+), OSR1(+)SIX2(−), OSR1(−)SIX2(+), and OSR1(−)SIX2(−) cell populations differentiated from the OSR1-GFP/SIX2-tdTomato reporter human iPSC lines revealed that the OSR1(+)SIX2(+) cell population,

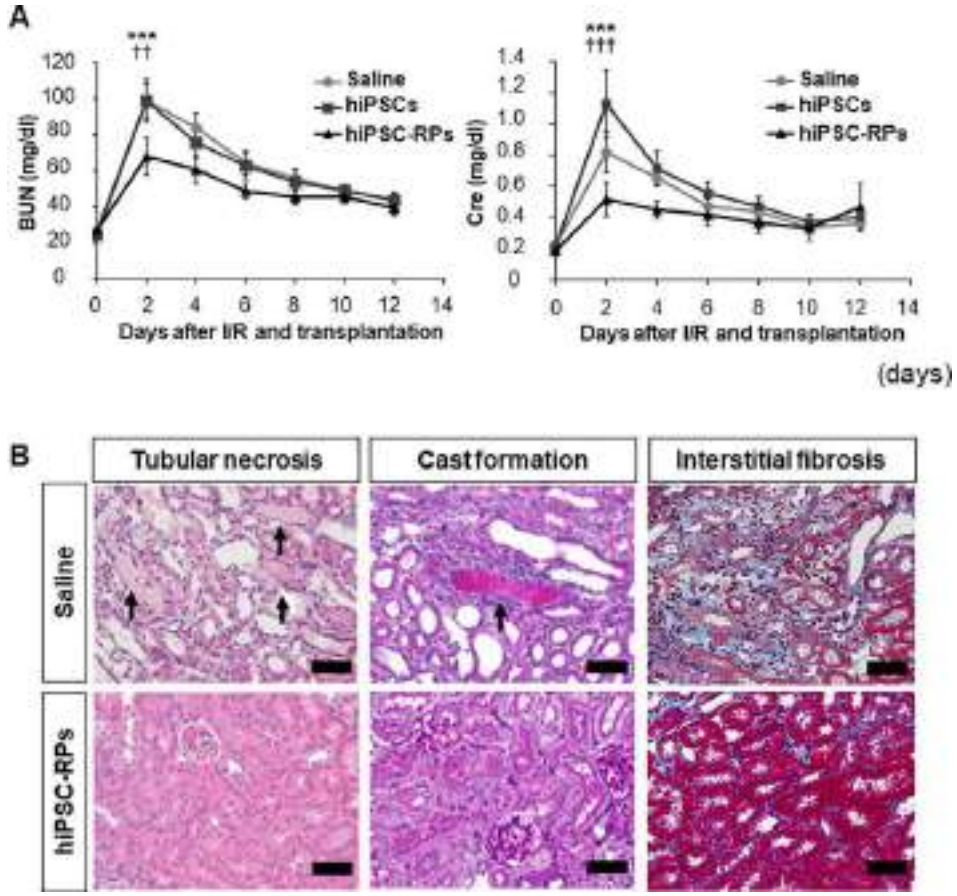

FIG. 10.2 Cell therapy. (A) Time course analysis of blood urea nitrogen (BUN) and serum creatinine (Cre) values in acute kidney injury (AKI) model mice induced by ischemia-reperfusion injury that received saline injection *(gray circles)* or renal subcapsular transplantation of undifferentiated human iPSCs (hiPSCs; *black squares*) or of human iPSC-derived renal progenitors (hiPSC-RPs; *black triangles*). $^{***}P < .001$ versus saline; $^{††}P < .01$ versus hiPSCs; $^{†††}P < .001$ versus hiPSCs. (B) Representative sectional images of host mouse kidneys that received the saline injection *(upper panels)* or the transplantation of hiPSC-RPs *(lower panels)* and stained with hematoxylin and eosin (HE; *left panels*), periodic acid-Schiff (PAS; *middle panels*), or Massoń's trichrome (MT; *right panels*) on day 12 after the ischemia-reperfusion injury and transplantation. *Arrows* indicate tubular necrosis in the *upper left panel* and cast formation in the *upper middle panel*. Scale bars, 20 μm. *Modified from Toyohara T, Mae SI, Sueta SI, Inoue T, Yamagishi Y, Kawamoto T, Kasahara T, Hoshina A, Toyoda T, Tanaka H, Araoka T, Sato-Otsubo A, Takahashi K, Sato Y, Yamaji N, Ogawa S, Yamanaka S, Osafune K. Cell therapy using human induced pluripotent stem cell-derived renal progenitors ameliorates acute kidney injury in mice. Stem Cells Transl Med 2015;4(9):980–92.*

which has developmental potential to form nephron structures, showed the most potent therapeutic effects on AKI [48]. Previous studies reported that the interstitial fibrosis that develops after AKI can cause CKD, suggesting the importance of preventing this interstitial fibrosis [49, 50]. Therefore, the mechanistic elucidation of the therapeutic effects of human iPSC-derived progenitor cells on interstitial fibrosis after AKI may contribute to the development of novel therapeutic strategies to prevent CKD. A detailed comparative analysis of the gene expression profiles of these populations might elucidate the molecular nature of the therapeutic factors.

Hoshina et al. screened monoclonal antibodies against 242 different cell surface markers in an attempt to purify human iPSC-derived OSR1(+)SIX2(+) NPC-like kidney progenitors without using reporter human iPSC lines toward clinical applications [51]. They found that the combination of four markers, CD9(−)CD140a(+)CD140b(+)CD271(+), enriches OSR1(+)SIX2(+) kidney progenitors differentiated from an OSR1-GFP/SIX2-tdTomato reporter human iPSC line. Furthermore, the transplantation of the human iPSC-derived purified CD9(−)CD140a(+)CD140b(+)CD271(+) kidney progenitors into renal subcapsules biochemically and histologically ameliorated the AKI induced by schemia-reperfusion injury with unilateral nephrectomy in immunodeficient mice [51].

The therapeutic benefits on AKI by embryonic kidney progenitors were also confirmed with mouse embryonic NPCs [43]. Li et al. showed that the renal subcapsular transplantation of mouse embryonic NPCs expanded in vitro using their NPSR medium improved the survival rate and ameliorated renal injury in cisplatin-induced AKI model

mice as measured by biochemical parameters, such as BUN and serum creatinine levels, and histological findings, including tubular dilatation and necrosis. The transplanted NPCs localized outside of the kidney cortex and did not integrate, suggesting once more that paracrine factors may produce beneficial effects. Furthermore, they confirmed the paracrine effects by intraperitoneally injecting the condition medium of mouse NPCs into cisplatin-induced AKI mice [43]. No reports have so far shown the therapeutic efficacy of cell therapies using human iPSC-derived NPCs in CKD animal models.

Kidney reconstruction

Several studies described earlier have generated nephron organoids containing glomeruli and renal tubules from human PSC-derived NPCs [20, 30, 33, 34], and one report generated collecting duct organoids from human iPSC-derived UB cells [40]. Other studies have demonstrated that, when the nephron organoids were transplanted into the renal subcapsular space of immunodeficient mice, the glomeruli in the organoids become vascularized and connect to the host blood stream [29, 52, 53]. Sharmin et al. also showed that human iPSC-derived podocytes in the transplanted nephron organoids accumulated numerous cell processes around fenestrated endothelial cells of the host mice [29]. van den Berg et al. confirmed functional glomerular perfusion by in vivo imaging human PSC-derived kidney organoids transplanted under the kidney capsule [52]. Wide-field electron microscopy demonstrated formation of the basement membrane, fenestrated endothelial cells and podocyte foot processes in glomeruli, and the polarization and segmental specialization of tubular epithelium after transplantation, indicating that functional vascularization induces glomerular and tubular maturation in vivo. Later, the same group assessed the glomerular function of human PSC-derived kidney organoids transplanted under the kidney capsule in immunodeficient mice by high-resolution intravital multiphoton imaging through a titanium imaging window implanted on top of the kidney organoids [53]. After vascularization, the human glomeruli displayed dextran and albumin size selectivity across their glomerular filtration barrier and proximal tubules uptook dextran, indicating functional filtration of the human PSC-derived glomeruli.

On the other hand, Taguchi and Nishinakamura generated mouse kidney organoids from NPCs and UB cells differentiated from mouse ESCs and stromal progenitors derived from mouse embryonic kidneys, in which glomeruli, renal tubules, and collecting ducts were interconnected [37]. Tsujimoto et al. succeeded in generating human kidney organoids from human iPSC-derived NPCs and UB cells in vitro, in which NPC-derived glomeruli and renal tubules and UB-derived collecting ducts were interconnected (Fig. 10.3A and B) [34]. When transplanted into the renal subcapsular space of immunodeficient mice, the human iPSC-derived glomeruli were connected to the host vasculature, and the connection of glomeruli to host blood stream was confirmed by intravital imaging using multiphoton microscopy (Fig. 10.3C–E). However, extensive branching of the UB was not found in the human organoids, suggesting stromal progenitors might be required as in mouse kidney organoids [37]. These kidney-like structures generated from human PSC-derived kidney progenitors are very small in size (several millimeters in diameter). The generation of larger kidney tissues are required for kidney transplantation.

For this goal, the research group headed by Hiromitsu Nakauchi has used interspecific blastocyst complementation [54, 55]. Here, wild-type PSCs are injected into the blastocysts of experimental animals genetically modified not to develop the target organs of interest, thus the entire organs are composed of cells derived from the injected PSCs. To demonstrate this approach, the group injected wild-type mouse and rat PSCs into Pdx1(−/−) (pancreatogenesis-disabled) rat and mouse blastocysts, respectively, and successfully generated an almost entirely PSC-derived pancreas interspecifically [54, 55]. Similar approaches have already been examined to generate an entire kidney [56, 57]. Usui et al. injected wild-type mouse ESCs into the blastocyst of knockout mice of *Sall1*, which is an essential gene for MM development [56]. Consequently, kidneys developed in the mice with MM-derived glomeruli and renal tubules that originated from the injected mouse ESCs, but the UB-derived collecting ducts, lower urinary tract, and vasculature were derived from the host mouse. However, when xenogeneic rat PSCs were injected into the blastocysts, rat PSC-derived kidneys were not obtained due to the poor contribution of rat PSCs to the mouse MM [56]. On the other hand, Goto et al. recently succeeded in generating mouse PSC-derived MM in rat by interspecific blastocyst complementation with the injection of mouse PSCs into the blastocyst of anephric Sall1(−/−) mutant rat [57].

Yamanaka et al. attempted to utilize the organ niche of later stage fetuses of different species for kidney organ regeneration [58]. The group established a combination system through which donor NPCs are precisely injected into the nephrogenic zone of Six2-Cre-inducible diphtheria toxin (DT) receptor transgenic mice, in which native NPCs of metanephros are conditionally eliminated by DT injection. They succeeded in removing Six2(+) NPCs in the nephrogenic niche of the host mice and replacing them with transplanted wild-type mouse NPCs in an ex vivo organ

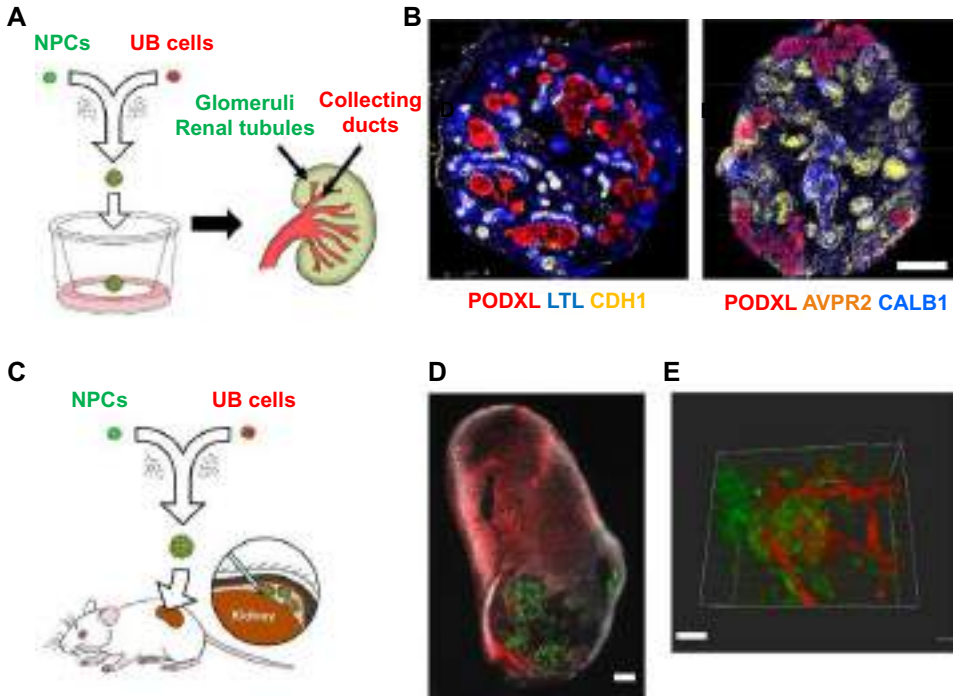

FIG. 10.3 Kidney reconstruction. (A) A schematic showing the strategy to reconstruct kidney structures from human iPSCs in vitro. (B) Triple immunostaining images of human iPSC-derived kidney organoids for markers of podocytes (PODXL), proximal tubules (LTL), and distal tubules and collecting ducts (CDH1; *left panel*) and for PODXL and markers of distal tubules and collecting ducts (AVPR2) and colleting ducts only (CALB1; *right panel*). Note that weak CDH1 signals were also found in parts of the LTL(+) proximal tubules in the *left panel*. (C) A schematic showing the strategy to reconstruct kidney structures from human iPSCs in vivo. (D) A low magnification image showing host kidney and human iPSC-derived kidney graft *(green)* after the administration of rhodamine B-conjugated dextran through the tail vein of the host mouse. (E) Intravital imaging with multiphoton microscopy after tail vein injection of rhodamine B-conjugated dextran showing the vessel lumens of host mice penetrate into the human iPSC-derived glomerulus structure *(green)*. Scale bars, 100 μm in (B), 500 μm in (D), and 40 μm in (E). *Modified from Tsujimoto H, Kasahara T, Sueta S, Araoka T, Sakamoto S, Okada C, Mae SI, Nakajima T, Okamoto N, Taura D, Nasu M, Shimizu T, Ryosaka M, Li Z, Sone M, Ikeya M, Watanabe A, Osafune K. A modular differentiation system maps multiple human kidney lineage from pluripotent stem cells. Cell Rep 2020;31(1):107476.*

culture setting. The transplanted NPCs developed into glomeruli and renal tubules that connected to host collecting ducts and further vascularized following transplantation. Furthermore, they succeeded in interspecifically generating rat nephrons by replacing host mouse NPCs [58]. Later, the same group developed a cell transplantation method through which mouse NPCs were accurately transplanted into the fetal kidney development area of E13-14 mouse embryos in uterine by incising the opaque uterine muscle layer but not the transparent amniotic membrane [59]. The transplanted NPCs contributed to the chimeric cap mesenchyme of donor and host NPCs that is connected to the host UB. This experimental approach also generated chimeric kidneys in which MM lineage cells were derived from donor PSCs while other kidney constituents including UB lineages were from host animals. Further studies should develop strategies to replace both the MM and UB lineage cells with donor PSC-derived cells or prevent immune rejection against host animal-derived cells after transplantation into kidney disease patients.

Disease modeling

The combination of iPSC and genome-editing technologies has facilitated the generation of disease-specific iPSCs, which possess the genetic predisposition that causes the disease. Disease modeling research has been vigorously carried out for numerous intractable disorders, in which disease-specific iPSCs are differentiated into the injured cell types to recapitulate the disease phenotypes in vitro [8]. Additionally, substantial advances in the directed differentiation of kidney lineages from human PSCs in the past decade has enabled the generation of kidney disease models (Fig. 10.4A).

Freedman et al. derived gene-edited homozygous *PKD1/2*-mutant human ESCs and succeeded in forming large, translucent, cyst-like structures mimicking renal cysts of autosomal dominant polycystic kidney disease (ADPKD) by

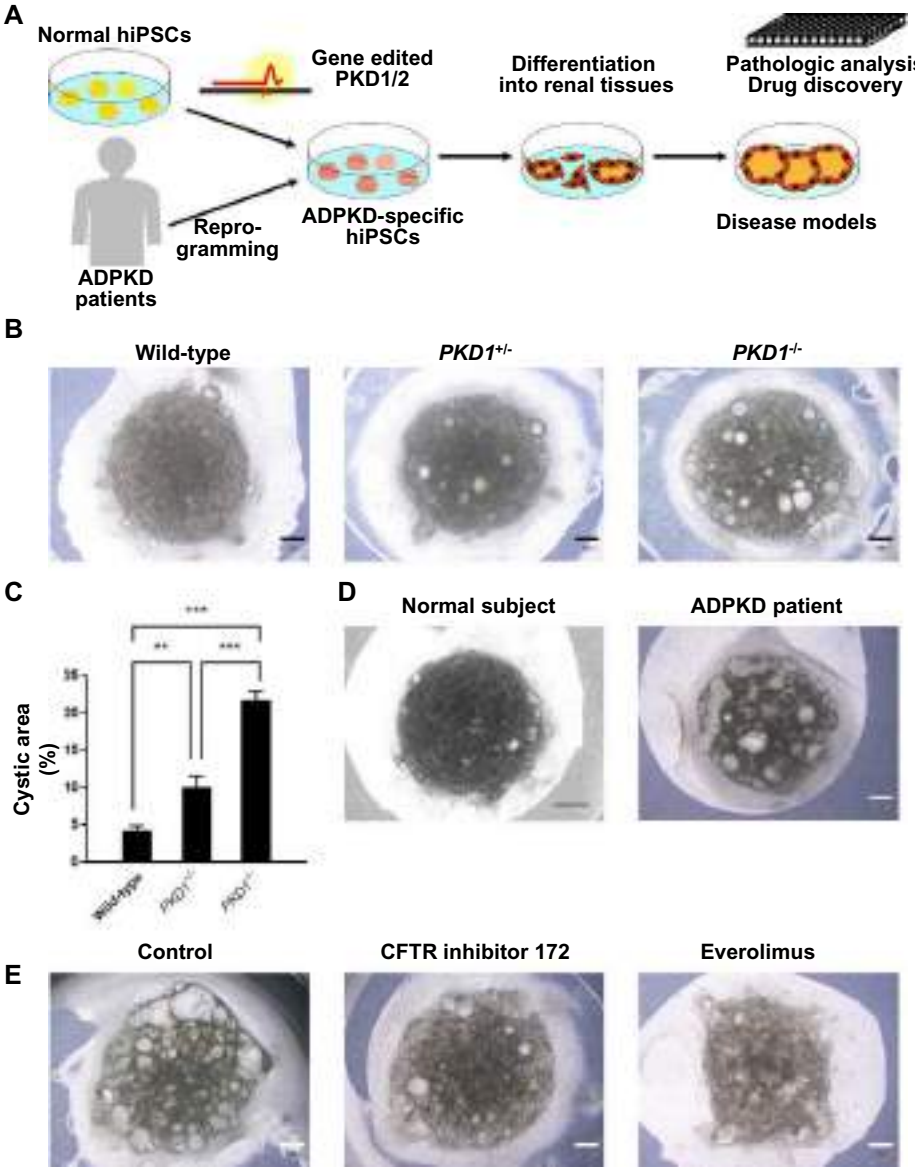

FIG. 10.4 Modeling ADPKD. (A) A schematic showing the disease modeling research for ADPKD. ADPKD-specific iPSCs can be generated by gene editing *PKD1/2* in healthy donor-derived iPSCs or reprogramming the somatic cells of ADPKD patients. These iPSC lines are differentiated into renal cells or tissues to generate disease models to analyze the disease mechanisms and conduct drug discovery. (B) Representative bright-field images of wild-type *(left panel)* and gene-edited heterozygous *(middle panel)* and homozygous *(right panel) PKD1*-mutant iPSC-derived nephron organoids after 7-day forskolin treatment. (C) Percentages of cyst areas in the nephron organoids in (B). Data are represented as the mean ± SE from three independent experiments with four replicates in each. $^{**}P < .005$ and $^{***}P < .001$ by one-way ANOVA and Bonferroni's method. (D) Representative bright-field images of normal subject-derived *(left panel)* and patient-derived *(right panel)* nephron organoids after 7-day forskolin treatment. (E) Representative bright-field images of patient-derived nephron organoids after 7-day treatment with forskolin alone (control; *left panel*) or combined with CFTR inhibitor 172 (100 μM; *middle panel*) or everolimus (10 μM; *right panel*). Scale bars, 300 μm in (B), (D, *right panel*), and (E) and 500 μm in (D, *left panel*). *Modified from Shimizu T, Mae SI, Araoka T, Okita K, Hotta A, Yamagata K, Osafune K. A novel ADPKD model using kidney organoids derived from disease-specific human iPSCs. Biochem Biophys Res Commun 2020;529(4):1186–94.*

adherent cultures of the differentiated mutant nephron organoids attached onto culture plates [60]. The group thereafter demonstrated that removing adherent cues by floating cultures substantially facilitates the cyst formation in the mutant organoids [61]. Furthermore, they established a fully automated high-throughput screening (HTS) platform for the differentiation and phenotyping of kidney organoids using automatic liquid-handling robots and high-content imaging analyzers to identify candidate drug compounds [62]. However, the group did not recapitulate the renal cyst phenotype of ADPKD in patient-derived or gene-edited heterozygous *PKD1*-mutant iPSCs. Shimizu et al. generated ADPKD-specific iPSCs by editing *PKD1* gene in healthy donor-derived iPSCs with the CRISPR-Cas9 system and

formed nephron organoids containing glomeruli and renal tubules from both gene-edited ADPKD-specific iPSCs and ADPKD patient-derived iPSCs previously generated by the same group [63, 64]. They found that patient-derived, heterozygous *PKD1*-mutant and homozygous *PKD1*-mutant nephron organoids all develop cyst structures by forskolin treatment (Fig. 10.4B–D). Furthermore, these cyst models can respond to candidate therapeutic compounds for ADPKD, such as an inhibitor of cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that facilitates transepithelial fluid secretion, and the mTOR inhibitors everolimus and rapamycin, suggesting that these models can be used for drug screening (Fig. 10.4E). Kuraoka et al. also recapitulated renal cystic phenotypes from gene-edited heterozygous and homozygous *PKD1*-mutant human iPSCs by differentiating the cells into nephron organoids and UB-like tissues and further treating them with forskolin or vasopressin [65]. Low et al. developed a platform to generate vascularized and segmentally patterned nephron organoids by modulating WNT signaling [66]. The group also derived disease-specific iPSCs from an autosomal recessive polycystic kidney disease (ARPKD) patient with compound heterozygous mutations of *PKHD1* gene and corrected one mutation of the iPSCs by the CRISPR-Cas9 system. They further differentiated both ARPKD patient-derived and corrected iPSCs into nephron organoids and successfully reproduced renal cyst formation by treatment with either forskolin or 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), which increased intracellular cAMP levels. Both thapsigargin, which releases intracellular Ca^{2+}, and CFTR inhibitors blocked the cyst formation in a dose-dependent manner [66].

For another ciliopathy, Forbes et al. identified compound heterozygous variants in *IFT140*, a gene previously associated with nephronophthisis (NPHP)-related ciliopathies, in a prospectively identified NPHP proband and her parents by trio whole-exome sequencing [67]. The group derived uncorrected proband iPSCs and isogenic gene-corrected iPSCs, which were differentiated to kidney organoids. Renal tubules in the proband kidney organoids showed shortened, club-shaped primary cilia, which was rescued by gene correction. A differential expression analysis of epithelial cells isolated from the proband and gene-corrected organoids suggested the downregulation of genes associated with apicobasal polarity, cell-cell junctions, and dynein motor assembly in proband epithelial cells, and Matrigel cyst cultures further confirmed a polarization defect in the proband epithelium [67].

Przepiorski et al. established a simple, inexpensive, and robust bioreactor-based method which generates kidney organoids in bulk from human PSCs and examined the roles of *HNF1β* gene in human nephrogenesis by generating kidney organoids from gene-edited HNF1β(−/−) iPSCs [68]. The mutant organoids showed a failure in the formation of proximal tubules and thick ascending limb segments like in *Hnf1β* knockout mice, as evidenced by the significant downregulation of marker genes for the segments by qPCR and immunostaining analyses [68].

Disease models for hereditary glomerular disorders have also been investigated [69]. Tanigawa et al. derived human iPSCs from a patient with congenital nephrotic syndrome caused by a missense mutation of the *NPHS1* gene, which encodes NEPHRIN, and resulting from impaired slit diaphragm formation in glomerular podocytes. The group reproduced the slit diaphragm formation process using human iPSC-derived nephron organoids and found impaired localization of NEPHRIN and impaired slit diaphragm formation. Genetic correction of the mutation restored NEPHRIN localization and phosphorylation, colocalization of other slit diaphragm-associated proteins, and slit diaphragm formation, suggesting that nephron organoids from disease-specific iPSCs identified the podocyte abnormalities at the initial phase of congenital nephrotic disease [69].

Regarding UB lineages, Taguchi et al. recapitulated some disease phenotypes of coloboma syndrome, which is one form of congenital anomalies of the kidney and urinary tract (CAKUT) caused by *PAX2* mutations and characterized by renal hypodysplasia and abnormalities, by generating UB-like tissues from gene-edited *PAX2* knockout human iPSCs [37]. Mae et al. generated a disease model for multicystic dysplastic kidney (MCDK), another form of CAKUT, using human iPSC-derived UB organoids [40]. They derived a heterozygous mutant human iPSC line for *HNF1β* gene, one of the causative genes of MCDK, by the CRISPR-Cas9 system and differentiated the cells into UB organoids. These HNF1β(+/−) UB organoids morphologically exhibited fewer budding regions and smaller RET(+) tip regions than wild-type UB organoids. Moreover, the mutant organoids lacked apicobasal polarity, as indicated by the reduced expression of the apical markers PRKCζ and EZRIN. These findings are consistent with those reported in MCDK model mice [70, 71]. However, the mutant organoids did not reproduce cystic structures, as the phenotype develops at later developmental stages than in the organoids [40].

Hurdles to overcome

Several directed differentiation protocols to induce human iPSCs into NPCs have been developed. However, in vitro expansion culture and purification methods for human iPSC-derived NPCs need to be developed toward clinical applications and practical use. Especially, expansion cultures using cheaper small molecule compounds would

contribute to lowering the cost of the NPC preparation. Although the directed differentiation of UB tissues has also advanced, one of the remaining challenges for the reproduction of UB lineage cells from human iPSCs is the poor induction of mature collecting duct cells. The directed differentiation of human iPSCs to functionally mature collecting duct cells, such as principal and intercalated cells, would contribute to examining the physiology of human collecting ducts and establishing disease models for multiple CAKUT forms, particularly cystic kidney diseases such as ADPKD and ARPKD. No reports have so far generated the third type of embryonic kidney progenitor cells, stromal progenitors, from human iPSCs.

The paracrine effects of renotrophic humoral factors secreted from NPCs are considered a main mechanism underlying the therapeutic benefits of cell therapy against AKI. Identifying the molecular nature and elucidating the mechanisms of action of these factors would facilitate the establishment of cell therapies. In addition, the development of human iPSC-based cell therapies that prevent the progression of CKD is expected. The functional integration and contribution of transplanted NPCs to host kidney tissues in addition to paracrine effects would produce more potent and long-acting therapeutic benefits.

For kidney reconstruction, the generation of large-sized kidney tissues is required for organ transplantation. The induced kidney tissues should also be connected to larger vessels and nervous systems when transplanted to the body. The formation of the lower urinary tract including the renal pelvis and ureter structures, into which numerous collecting ducts gather, is also required to generate functional kidneys. One promising approach is to utilize an organ niche environment of experimental animals including genetically manipulated pigs. Several previous reports have generated kidney tissues by seeding NPCs inside scaffolds composed of the extracellular matrices that remain after kidney decellularization [72]. Another study reported 3D kidney tissues artificially generated with a 3D printer [73]. These techniques may contribute to the generation of large-sized kidney tissues from human iPSCs. Another issue is the immaturity of kidney organoids generated from human iPSCs. Functionally mature kidney tissues would contribute to disease models for intractable kidney disorders in addition to kidney transplantation research. One possible strategy regarding maturity is vascularization and the addition of physical stimuli, such as blood or urinary flow and shear stress, to the human iPSC-derived kidney structures. Organ-on-a-chip technology has already been used to prepare human iPSC-derived mature nephron structures for drug and toxicology screening by combining kidney organoids with a vascular network consisting of endothelial cells [74].

Finally, HTS systems, which are applied to iPSC-based disease models already established for intractable kidney disorders such as ADPKD, will help identify novel drug compounds against kidney diseases.

Conclusions

During the last decade, kidney regeneration research using human iPSCs has substantially advanced and directed differentiation methods to induce human iPSCs into embryonic kidney progenitors, NPCs and UB cells, have been established. Kidney organoids containing nephrons and collecting ducts can be differentiated from these progenitors, which enables the generation of kidney disease models for pathological analysis and drug screening. The development of novel therapeutic strategies using iPSC-based technology, such as cell therapies, the transplantation of reconstructed kidneys, and drug discovery, is expected during the next decade.

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CHAPTER

11

Nephroprotective effect of urine-derived stem cells for renal injury

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Introduction

Renal insufficiency is a common clinic disorder and can develop suddenly (acute) or over the long term (chronic). Various causes of renal dysfunction include ischemia, toxicity, metabolic disorders, chronic inflammatory, and aging. Chronic renal dysfunction can be profound and may accelerate progression to end-stage renal diseases. Currently, hemodialysis and renal transplantation are both effective treatment modalities for chronic renal failure. However, hemodialysis is expensive with many potential complications, while renal transplant as a treatment modality is limited by the number of organ donors available. Stem-cell-based therapy provides the alternative in the treatment of kidney tissue injuries or diseases. Several types of stem cells have been used in preclinical animal models to evaluate the nephroprotective potential in repairing renal tissue. These stem cells include pluripotent stem cells such as induced pluripotent stem cells (iPSC) [1], multipotent mesenchymal stem cells (MSC) from different tissues, including fetal kidney [2], bone marrow [3–7], adipose [8], hair follicle [9], and amniotic fluid [10]. Ideal cell candidate for cell therapy in the treatment of kidney dysfunction should be the cells that (i) are easily accessible from a patient's own cells for the potential use in personalized medicine; (ii) could survive and exert the reparative function under disease-stress after implantation; (iii) are able to potentially inhibit inflammatory response, fibrosis, and oxidative stress for therapeutic purposes; (iv) are safe to use in vivo without any risk of oncogenicity; and (v) are able to give rise to podocytes and renal tubule epithelial cells for the potential use for in vitro renal disease modeling.

We are the first to demonstrate that renal stem cells exist in urine from human [11–13] and animals [14, 15], named as "urine-derived stem cells" or USC. These cells possess stem cell characteristics, i.e., clonogenicity, robust cell proliferation capacity, multipotent differentiation, proangiogenic paracrine effects, immune-modulatory properties, and easily induced pluripotent stem (iPSC) cells [16, 17]. USC consistently express renal progenitor cell markers (CD24, CD44, and CD 133) and MSC/pericyte markers (CD73, CD91, and CD105) and some key stem cell surface markers (SSEA4 CD117 and CD146), but not hematopoietic stem cell markers (except for MHC-1), endothelial cell markers (CD31), or human leukocyte antigen (locus) DR (HLA-DR) [11–13]. Compared to other MSC, USC have several advantages [18]: (i) they can be collected using a simple, safe, low-cost, and noninvasive procedure; (ii) they display telomerase activity so that they are able to generate more cells; (iii) they more efficiently differentiate into cell lineages in urinary tract system, such as podocytes, renal tubular epithelial, endothelial, urothelial, smooth muscle cells; (iv) USC showed higher myogenic, neurogenic, and endogenic differentiation rate, compared to adipose tissue stem cells [19]; (v) USC starts from one cell clone on one well in the initial culture, which generate the pure cell population for further studies; (vi) to isolate cells from the tissues, enzymes are required to digest the tissue and separate cells from the other cells, but this digesting tissue process affects cell viability. Cell viability of USC is better protected [12, 20] because isolation of USC does not require tissue dissociation procedures (such as digestive enzymes).

Although there are several cell types in urine, USC culture system can easily isolate stem cells from other somatic cells [11]. Although stem cells are a very small cell population in urine (4–7 USC clones/100 mL urine), one USC clone generates up to one million cells in vitro at the early passage (< p5) within 4 weeks [13, 20]. After optimizing our

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methods, 100–140 USC clones/24 h urine were consistently obtained from each individual [20]. Thus, a 24-h urine sample can provide ample number of cells (> 100 × 10 [6]) in 3 weeks for the purposes of cell implantation. There are two types of USC: one expressing telomerase activity (USC-TA$^+$), and other not expressing telomerase activity (USC-TA$^-$). Up to 75% of USC collected from middle-aged donors expressed telomerase activity and retained telomere length [21] (USC-TA$^+$) and the rest are USC-TA$^-$. These USC-TA$^+$ possessed higher proliferative capacities and were maintained for up to 67 population doublings after 16–20 passages, indicating that a single USC can generate up to 2^{67} cells within 14 weeks [13], while USC-TA$^-$ retain about 30 population doublings after 8 passages. Importantly, although most USC display telomerase activity, these cells do not form teratomas or tumors after subcutaneous implantation or under the renal capsule for up to 3 months, indicating that USC-TA$^+$ have high proliferation potential and also safe source for cell therapy [21, 22]. Thus, USC as an optimal cell source hold potent regeneration capacity for cell-based therapy in the treatment of kidney diseases, compared to other adult stem cells.

As a renoprotective agent, USC play an important role in promoting renal tissue remodeling and preventing the end-stage renal failure. More evidences demonstrate that mechanism of renoprotective effect of adult stem cells is paracrine effects to stimulate endogenous cells participating in tissue regeneration instead of cell differentiation. Kidney is a highly complex organ, composed of about 43 cell types within a unique structural framework [23]. USC secrete several types of biofactors, such as angiogenic growth factors, antiinflammatory factors, antifibrotic factors, antiapoptotic factors, antioxidative factors (Table 11.1). Despite giving rise to different renal cell types for the potential use in renal diseases modeling, no data showed that USC differentiate into renal cells after implantation in vivo. In this chapter, we focus on nephron-protective benefits of USC via trophic effect for renal injury in preclinical experiments.

Origin of urine-derived stem cells

The locations of endogenous renal stem cells are controversial. Several segments of the nephron have been considered, including glomerular, renal tubules, and papilla [18]. However, increasing evidences have demonstrated that Bowman's capsule contains a subset of parietal epithelial cells as source of adult stem cells in the kidney [24], displaying self-renewal properties and the potential to give rise to podocytes and proximal tubular cells. Traditionally, parietal epithelial cells are commonly obtained from kidney tissue biopsies, but this approach can cause high risk of complications. It is also difficult to isolate pure parietal epithelial cells without any cell type contamination.

TABLE 11.1 Biological characteristics and nephroprotective mechanisms of human urine-derived stem cells.

Telomerase activity (TA) expression	
Number of USC clones	4–7 USC clones/100 mL urine; about 140 USC clones/24 h urine
TA expression	Two types of USC: USC$^+$ (population doubling up to 68) and USC^{TA-} (population doubling at around 30)
Passage effect of TA expression in USC	TA expression decreases in USC with increased passages, which prevents tumor formation with unlimited proliferation features
Aging effect of TA expression in USC	3/4 of USC^{TA+} at the ages of 20–40 years old; 1/2 of USC $^{TA+}$ at the ages of 50 years old
Trophic effect	
Secrete factors or exosomes	VEGF, HGF, BMP-7, IGF1, prostaglandin E2
Antiinflammatory	Th2, T regulatory cells, IL10
Immunomodulation	Th1, lymphocytes, macrophages, IL-6, IL-122, TNF-α, IFN-ϒ, MCP PDGF-β, BAFF
Antifibrosis	MPP9, CTGF, TGF-β1, Type1 collagen, and PAI-1
Antiapoptosis	EGF, Akt, and Bcl-2
Antioxidative stress	From ROS to RNS
Safety	No teratoma or tumor were observed in vivo after a long-term follow-up

Abbreviations: *USC*, human urine-derives stem cells; *TA*, telomerase activity; *USC^{TA}+*, USC expressing telomerase activity positive; *USC^{TA-}*, USC expressing telomerase activity negative; *IL*, interleukin; *TNF*, tumor necrosis factor; *IFN-γ*, interferony; *MCP*, monocyte-chemotactic protein; *PDGF-β*, platelet-derived growth factor-*β*; *BAFF*, B cell activating factor; *CTGF*, connective tissue growth factor; *TGF-β*, transforming growth factor-*β*; *PAI-1*, plasminogen activator inhibitor1; *EGF*, epithelial growth factor; *VEGF*, vascular endothelial growth factor; *HGF*, hepatocyte growth factor; *IGF-1*, insulin-like growth factor; *BMP-7*, bone morphogenic protein-7; *ROS*, reactive oxygen species; *RNS*, reactive nitrogen species; *MMP9*, metalloproteinase-9.

A series of our studies demonstrated that USC are from the upper urinary tract system, instead of bladder: (1) USC isolated from urine obtained from the upper urinary tract during renal surgery are similar to voided USC in morphology, cell phenotypes, growth pattern, and differentiation capacity [12]; (2) USC from women who had received transplanted kidneys from male donors contain the Y chromosome [13, 25]. Both studies indicated that the USC originate from upper urinary tract. Next, we demonstrated that USC express cell markers, CD24, CD44, CD133, and CD146 at a rate similar to that expressed in parietal epithelial cells in glomerulus [13, 25], while bladder and ureter urothelial and smooth muscle cells do not. Another research team recently has confirmed that renal progenitor cells exist in human urine samples [26], supporting our initial findings about the locations of USC. Interestingly, renal fetal progenitor cells can be found in human amniotic fluid as a subpopulation derived from the fetal kidney [27], displaying the nephroprotective effect in acute ischemia reperfusion model.

In this chapter, we summarize the progresses in several related topics on nephroprotective benefits of USC therapy, including some of our data in acute and chronic renal insufficiency animal models. USC provide a source not only to treat different renal diseases but also to study the mechanisms of renal diseases caused by injury to parietal epithelial cells.

Treatment of acute renal insufficiency

Acute renal insufficiency is a common clinical disorder with high rates of morbidity and mortality. Stem cell therapy is emerging as a promising therapeutic strategy for acute renal insufficiency. Several studies on human USC [28] contribute to the repairing acute renal injury in rat models induced by ischemia or anticancer drug (i.e., cisplatin).

Tian and et al. [28] reported benefits of human USC on recovery of renal histological structures in a rodent model of ischemia/reperfusion (I/R)-induced acute renal injury. The implanted USC significantly improved renal function by reducing serum creatinine and blood urea nitrogen levels, decreased tubular injury score, and decreased number of apoptotic cells. Compared with the control group, the mRNA expression levels of the antiinflammatory factors (interleukin (i.e., IL-10 and transforming growth factor-β1)) were significantly upregulated, while expression levels of the proinflammatory factors (i.e., interferon-γ and IL-1β) were significantly reduced after USC implantation. In addition, USC labeled with green fluorescent proteins were detected in the tubular epithelial lining. These findings suggested that USC promote kidney repair and improve function after ischemic renal injury in a rat model.

In other studies [27], human fetal renal progenitor cells can be found in amniotic fluid as these cells are derived from urinary tract system. These fetal renal progenitor cells highly expressed CD24, CD117, SIX2, and PAX2 and showed the nephroprotective potential in acute ischemia reperfusion (I/R) model and prevent late stage fibrosis. Serum creatinine level significantly reduced 24 h after stem cells were delivered intraarterially in rats with renal I/R injury, less tubular necrosis, less hyaline cast formation, higher proliferation index, less inflammatory cell infiltration, and less myofibroblasts at 48 h. Less fibrosis and proteinuria in the treated rats 2 months after stem cell injection.

Therapeutic effects of USC also demonstrated in other causes of acute kidney insufficiency, i.e., cisplatin-induced renal injury model [29]. In vitro, rat tubular epithelial cell line (NRK-52E) was incubated with cisplatin to induce cell damage, cell cycle arrest occurred in the S phase and became worse with increasing concentrations of cisplatin. Following coculture with USC, the cisplatin-induced NRK-52E cells showed higher cell viability and a lower apoptosis ratio than those of the control group, and cell cycle arrest was mitigated. In a rodent model of cisplatin-induce renal injury, the expression levels of proinflammatory cytokines (TNF-α and IL-6) and apoptosis-related proteins (BAX and cleaved caspase-3) were downregulated after USC therapy. In addition, histological and ultrastructural abnormalities improved, while apoptosis and inflammation were inhibited in USC-treated animals, compared to PBS-treated control. Finally, presence of GFP-labeled USC was noted in renal tissues in the USC-treated animals 4 days after implantation.

Treatment of chronic renal failure

Chronic kidney disease (CKD) is a significant medical problem due to recurrent or progressive injuries in glomeruli, tubules, interstitium, and/or vasculature. Several animal models represent various elements of human CKD to test the efficacy of cell therapy. Human USC have been evaluated in three CKD rodent models: diabetic nephropathy, and dual injuries of renal ischemia plus administration of gentamicin, and the aging-related chronic renal damage.

Diabetic nephropathy models

Diabetic nephropathy is one of the most serious complications in patients with diabetes. At present, there are no satisfactory treatments available for diabetic nephropathy. Stem cells are currently the main candidates for the development of new treatments for diabetic nephropathy. We and other investigators studied the renal protective potential of USC [22, 30] or USC-secreted exosomes [31] on diabetic nephropathy at an early stage, diabetics type 1 or type 2 rodent models induced by streptozotocin alone [30, 31] or combination of streptozotocin with high fat diet [25].

Renoprotective benefits of human USC were observed in multiple involved organs in vivo, such as pancreatic islets, the myocardium, the renal glomerulus, and the bladder detrusor in diabetic rat models [25]. Type 2 diabetic rat models were induced by means of a high fat diet and intraperitoneal injection with streptozotocin. Human USC isolated from voided urine were administered via tail veins. The functional changes of pancreatic islets, left ventricle, glomerulus, and bladder micturition were assessed by means of insulin tolerance tests, echocardiography, urine biochemical indexes, and cystometry. The histologic changes were evaluated by hematoxylin and eosin staining, Masson's trichrome staining and TUNEL staining. Treatment with USCs significantly alleviated the histological destruction and functional decline. Although the USC treatment did not significantly decrease fasting blood glucose, the fibrosis and apoptosis of the myocardium, glomerulus, and detrusor muscle were significantly inhibited. This study indicates that administration of USC may be useful for the treatment of the complications of diabetics, including diabetic nephropathy. This renoprotective effect of USC on diabetic nephropathy was also observed in type I diabetic mouse model [30]. The implanted USC significantly improved renal function, inhibited oxidative stress, as well as reduced renal fibrosis and histological damage after cell injection at three times.

A recent study showed that exosomes derived from USC (USC-Exo) reduced kidney injury in in vivo diabetic type I model [31]. USC-Exo were approximately 50–100 nm spherical vesicles, and the specific markers included CD9, CD63, and CD81. In vitro, USC-Exo could reduce podocyte apoptosis induced by high glucose. Enzyme-linked immunosorbent assay showed that USC-Exo contained the angiogenin and bone morphogenetic protein-7, which promote vascular regeneration and cell survival. In vivo, USC-Exo significantly reduce the urine volume and urinary microalbumin excretion, prevent podocyte and tubular epithelial cell apoptosis, suppress the caspase-3 overexpression, and increase glomerular endothelial cell proliferation after weekly tail intravenous injection of USC-Exo or control until 12 weeks.

A more recent study further demonstrated that exosomal microRNA-16-5p from human USC ameliorates diabetic nephropathy through protection of podocyte [32]. The miR-16-5p is predicted to target vascular endothelial growth factor A (VEGFA) based on data retrieved from several bioinformatics databases. Notably, dual-luciferase report gene assay provided further verification confirming the prediction. This study results demonstrated that high glucose stimulation inhibited miR-16-5p and promoted VEGF-A in human podocytes. miR-16-5p in USC was transferred through the exosome pathway to high glucose-treated podocytes. The viability and apoptosis rate of podocytes after high glucose treatment together with expression of the related factors were subsequently determined. The results indicated that miR-16-5p secreted by USC improved the podocyte injury. In addition, VEGF-A silencing could also ameliorate HG-induced podocyte injury. In in vivo experiments, USC exosomes containing overexpressed miR-16-5p injected to diabetic rats via tail vein conferred protective effects on podocytes in diabetic rats. Taken together, this study revealed that overexpressed miR-16-5p in USC exosomes could protect podocytes against high glucose and suppress VEGF-A expression and apoptosis in podocytes, providing fresh insights for novel treatment of diabetic nephropathy.

Gentamicin + ischemia model

Drug-induced nephrotoxicity can occur in patients with preexisting renal dysfunction or renal ischemia, potentially leading to CKD and end-stage renal disease (ESRD). Prompt treatment of CKD and the related side effects is critical in preventing progression to ESRD. Recently, we studied the therapeutic potential of USC in treating CKD induced by nephrotoxic drugs in combination with renal ischemia [33]. An athymic rat model of CKD was induced by creating an ischemia-reperfusion injury and gentamicin administration. In total, 24 rats were divided into three groups: PBS-treated group ($n=9$), USC-treated group (n = 9), and sham group with age-matched control animals ($n=10$). Cell suspension of USC (5×10 [6]/100 μL/kidney) or phosphate buffered saline (PBS) was injected bilaterally into the renal parenchyma 9 weeks after CKD model creation. Our results showed that renal function, measured by glomerular filtration rate (GFR) and serum Cr in USC-treated group, was significantly improved compared to PBS-treated rats 12 weeks after cell injection. The degree of glomerular sclerosis and atrophic renal tubules, the amount of fibrosis, and monocyte infiltration significantly decreased in USC-treated group compared to the PBS group. The

level of TGF-ß1 expression in renal tissues was also significantly lower in the PBS group, while the level of SOD-1 expression was significantly elevated in the USC group, compared to PBS group. We concluded that nephroprotective effect of USC on renal function is accomplished via antiinflammatory, antioxidative stress, and antifibrotic activity in a dual-injury CKD rat model. This provides an alternative treatment for CKD in certain clinical situations, such as instances where CKD is due to drug-induced nephrotoxicity and renal ischemia.

Aging-related CRF

Kidney function declines with age and the elder people are more at risk of some kidney diseases. About 40% of older adults (> 60 years old) develop renal dysfunction, compared to 8.9% of young people (20–39 years old) with renal diseases [34]. Cell-based therapy is a promising alternative method to restore renal function in the treatment of chronic renal insufficiency. Our pervious study demonstrated that human USC prompted renal tissue remodeling in a rodent model of age-related kidney insufficiency [35]. In this study, 18 male Sprague Dawley rats (50–62 weeks old) with renal insufficiency (increased levels of urine protein and serum creatinine) were divided into three groups (G). G1, animals received five intravenous cell implantations of 2 x 10^6 cells/0.2 mL serum-free medium/rat/time weekly; G2, as in G1 but intraperitoneally delivered; G3, as in G1 but serum-free medium alone. Healthy male SD rats at the same age were used as controls. The results showed that cultured human USC secreted > 20 trophic factors, including high levels of matrix metalloproteinase 9, which is involved in extracellular matrix degradation. Histologic and immunohistochemical staining showed that ED1 (inflammatory marker), alpha-smooth muscle actin (myofibroblast marker), and collagen deposition were significantly reduced within the medulla or cortex in treated rats with renal insufficiency vs. controls. G1 and G2 rats showed significantly inhibited inflammation and fibrosis in interstitial tissue, and reduced collagen deposits around renal vessels and the basement membrane of renal tubule and glomerular tissue, compared to G3. In conclusion, therapeutic use of USC can decrease the influx of inflammatory macrophages, interstitial fibrosis, and collagen deposition through their paracrine effects. Our unpublished data show that normal USC can be obtained from the seniors and still hold the potential to self-renew or repair the aging kidney.

Besides treatment of renal diseases, the implanted USC can be used in the most tissue repairs, particularly in genitourinary tract system. Our studies demonstrated that USC restored erectile function in a rodent model of diabetic erectile dysfunction [36], and improved urethral sphincter function in a rat model of vaginal distention injury [37]. Our procedures with USC have been successfully repeated by others [19, 31, 38–50]. They used human USC for kidney [31, 38, 51], bladder [47], cardiac tissue [22], corpora cavernosa [52], bone [45, 53], lungs [49], skin [54], neuronal [55], and other types of tissue regeneration [53, 54]. Importantly, our more recent studies show that up to 30% of USC from the patients with chronic diseases (such as diabetic nephropathy) possess normal regenerative function in cell proliferation, differentiation, and paracrine factor secretion (unpublished data). In conclusion, USC conveniently obtained by a noninvasive, safe, reproducible, and low-cost approach would be highly desirable as an alternative cell source to improve various tissue regeneration, particularly in patients with kidney diseases.

Future directions

Acute renal injury and chronic kidney disease pose growing problems. The benefits of USC-based therapy in renal injury and diseases mostly confined to small animal models, and so far, the clinical studies have been limited to the anecdotal patients' reports. These preclinical studies of stem cell therapy provide important information to set up the fundamental basis for clinical practice. Before use in clinical practice, the strategies of USC-based therapy need to be optimized. Several key factors should be addressed in cell preparation [56], including cell passages, culturing USC with endogenous or exogenous growth factors, preconditioning of USC with hypoxia, growth factors, or conditioned medium, using various controlled release systems to deliver growth factors with hydrogels or microspheres to provide optimal interactions of stem cells and their niche. In addition, several approaches for cell delivery may affect the outcomes of cell therapy, including the appropriate routes of cell administration (systemic, intravenous, or intraperitoneal vs. local administration), timing for USC therapy (immediate vs. a few days after injury), single injection of a large number of USC vs. multiple smaller number of USC injections, a single site for injection vs. multiple sites. To date, no apparent side-effects have been noted in animal experiments; however, like with the use of other stem cell types, the efficacy and safety of USC therapy in patients should be closely monitored and evaluated when these cells are used in clinical applications.

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CHAPTER

12

Amniotic stem cells and their exosomes

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Introduction

Over the past decades, treatment for renal failure has changed little. Except for kidney transplantation and dialysis, few therapeutic options are effective against these intractable diseases. Recent years, cell-based regenerative medicine is becoming a promising application to ameliorate kidney injury and accelerate repair. People are even pursuing to generate new renal tissues from stem/progenitor cells [1]. It has been verified that various kinds of stem cells, such as mesenchymal stem cells (MSCs) [2, 3], adipose stem cells (AdSCs) [4], amniotic fluid stem cells (AFSCs) [5], renal progenitors (RPs) [6], and so forth, can restore renal function and stimulate renal repair in preclinical models of acute and chronic kidney injury. Nevertheless, ethical and technological issues have limited the clinical applications of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) [7]. Recently, scientists have focused their attention on a versatile cell population, the perinatal stem cells, including cells from the entire placenta, the amnion, the amniotic fluid, the umbilical cord blood, and the Whartoń s jelly. Although perinatal stem cells are classified as adult stem cells, they express markers intermediate between adult stem cells and ESCs, possessing pluripotency as ESCs and immunomodulatory properties as adult stem cells both in vitro and in vivo [8, 9].

The amnion, also called amniotic membrane (AM), is the innermost extraembryonic membrane that surrounds the fetus and contains the amniotic fluid. This avascular thin membrane is considered a part of the organ placenta in a pregnant woman and provides mechanical and immune protection throughout gestation [10]. Two types of cells, named amniotic epithelial cells (AECs) and amniotic mesenchymal stem cells (AMSCs), have been isolated from amnion via established protocols [11]. Mounting evidence has shown that both populations of cells possess the characteristics of embryonic stem cells, which have the potential for differentiating into all three germ layers and immunomodulatory properties as adult stem cells [12]. Amnion-derived stem cells overcome the limitations in other stem cells, such as ethical considerations in ESCs, difficulties of procurement from adult tissues, and concerns with respect to tumorigenicity [13], therefore, making them a precious candidate to be a reliable source for regenerative medicine. They now have been widely used in the basic research of numerous diseases and injuries including kidney insults [14].

As will be reviewed in this chapter, the majority of findings illuminate that amniotic stem cells (ASCs) enhance the endogenous tissue regeneration or repair by paracrine or endocrine-mediated signaling rather than transdifferentiating into injured renal cells. Almost all living cells can secrete extracellular vesicles (EVs) as part of their normal physiology or acquired abnormalities [15]. Exosomes are one of categories of EVs with an average diameter of 100 nm [15]. It has been well established that exosomes have the potential for disease diagnosis and therapy by delivering proteins, metabolites, nucleic acids, and microRNAs (miRNA or miR) into recipient cells and alter their biological response [16–18]. Albeit a few clinical trials currently accomplished, exciting basic and preclinical discoveries have demonstrated the abilities of ASC-derived exosomes in restricting injury and enhancing repair [19, 20]. The emerging body of evidence about the effects of ASC-derived exosomes in renal injury and repair has open the doors to novel options in regenerative medicine with the increasing understanding of cellular pathophysiology in acute kidney injury (AKI) and the driving forces to chronic kidney disease (CKD).

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The amniotic stem cell

The origin of amnion and amniotic stem cells

The placenta connects fetus with mother to conduct material exchange including waste elimination, nutrient uptake, and gas exchange [21]. Although the placenta is normally discarded as medical waste after birth, it has been used as Chinese medicine for medical purpose since ancient times. Anatomically, the human placenta is composed of the amnion, the chorion frondosum, and the decidua. The amnion lines the amniotic cavity and is the innermost layer of the placenta [8]. AM can be easily obtained from placenta with properties of antimicrobial, antiangiogenic, antitumorigenic, and mechanical characteristics which make it an attractive biomaterial [22].

The amnion contains a thin (about 0.02–0.5 mm in thickness), avascular, smooth layer of epithelial cells, and connective tissues without nerves and lymph. It is derived from the fetal ectoderm through the cavitation of the embryonic knot [23]. During the early stage of blastocyst implantation (approximately at day 8–9 postconception), the outer cells (trophoblast) of the blastocyst differentiate into two layers and migrate into the endometrial stroma. The embryoblast (also known as the inner cell mass) also differentiates into two layers: the epiblast and the hypoblast. Epiblast is the origin of all three germ layers and eventually forms the embryo. Subsequently, some small cells located on the inner surface of the epiblast cluster with the enlarged space arise from embryoblast and neighboring trophoblastic cells. These amniogenic cells (amnioblasts) are the forerunners of the amniotic epithelium (AE). The cleft separating amnioblast from the embryoblast is the early amniotic cavity [14, 24, 25]. Concomitantly, the amniotic cavity enlarges and the layer of extraembryonic mesenchyme around the AE layer develops into the amniotic connective tissue. During the first trimester, the amnion projects into the former blastocyst (also termed extraembryonic coelom at this stage) and extends along the dorsal surface of the embryo. The sac gradually grows and elongates to the ventral surface of the embryo as pregnancy progresses, folding and ensheathing the connecting stalk to form the umbilical cord. The extraembryonic coelom is obliterated when the AM finally comes into contact with the primary chorionic plate at the end of the first trimester [26].

The AM can be separated into placental amnion, umbilical amnion, and reflected amnion based on the different attachment surfaces of the placenta, umbilical cord, and the chorion laeve, respectively [24]. Histologically, there are five layers distinguished from the amnion, including an epithelial cells monolayer, an acellular basement membrane layer, a compact layer, a fibroblast layer, and a spongy layer, and the latter three layers can be collectively named the stromal layer (Fig. 12.1) [27, 28]. Among these structures, the epithelial monolayer consists of flat, cuboidal, and columnar cells contacting with amniotic fluid directly. The compact layer is made up of connective tissue to maintain the mechanical integrity of the amnion. By now, two different stem and stem-like cell populations have been isolated from the amnion: AECs and AMSCs (which will be described in greater detail later), which are collectively

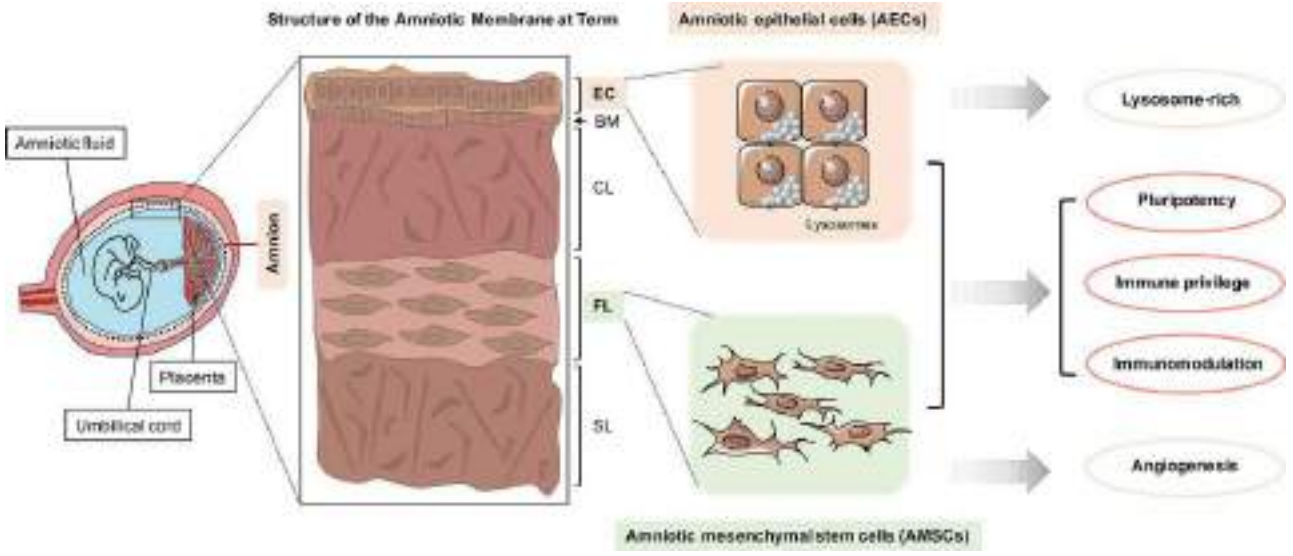

FIG. 12.1 The structure of amnion and characteristics of amnion stem cells. The cellular layers of the amniotic membrane are shown in detail. Different types of amniotic stem cells are listed for each cellular layer (right). Five properties of amniotic stem cells are shown on the right: pluripotency, immune privilege, immunomodulation, abundance of lysosomes, and angiogenesis. *EC*, epithelial cell monolayer; *BM*, basement membrane; *CL*, compact layer; *FL*, fibroblast layer; *SL*, spongy layer.

called ASCs (unless specifically noted, the ASCs in this chapter refer to human-derived). Over the past decades, an emerging interest in ASCs for clinical applications mainly taken advantage of the immune-privilege of these cells [29]. With the unique advantages discovered in basic research over other types of stem cells, ASCs now become a precious candidate to be widely studied in numerous diseases and injuries such as liver diseases, cardiovascular diseases, musculoskeletal diseases, neurological disorders, and ocular surface diseases [14]. Of note, these cells are particularly advantageous in the treatment of kidney injury, since they are safer and exert more plasticity than other adult counterparts.

Amniotic epithelial cells

Unlike other parts of placenta, the AE is derived from the pluripotent epiblast as early as the 8th day after fertilization [30]. This event occurs prior to gastrulation, a phase determining cell fate in early embryonic development, and thus, the AECs might retain some characteristic markers of ESCs [31]. There are four unique and advantageous properties of AECs: (a) pluripotency, (b) immune privilege, (c) immunomodulation, (d) abundance of lysosomes [32], which make them promising in usage for clinical cell therapy (Fig. 12.1).

AECs can be isolated via trypsinization and cultured easily [33]. One human placenta can provide sufficient counts of about 100 million (1×10^8) AECs. Freshly isolated AECs are circular medium-sized cells, with a central or eccentric nucleus, one or two nucleoli, and abundant cytoplasm [34]. Miki et al. described the stem cell characteristics of AECs in 2005, including expression of the stem cell-specific surface antigens similar to ESCs such as stage-specific embryonic antigen (SSEA)-3 and -4, as well as tumor rejection antigen (TRA) 1–60 and 1–81 but no expression of SSEA-1 [31]. However, these markers gradually disappear with the progression of gestation, only some of these stem cell marker-positive cells can be retained in term placenta AE. Flow cytometric analyses indicate that the ratios of stem cell marker-positive cells in the term AECs are varied (e.g., SSEA-3 and TRA1–60, 5%; TRA1–81, 10%; SSEA-4, 50%) [35]. Additionally, not all AECs coexpress these four antigens. Bryzek et al. further found that only about 4% of AECs coexpress SSEA-4, TRA1–60, and TRA1–81 antigens [36], which manifest that AECs are a heterogeneous cell population. Along with surface antigens, AECs express transcription factors of pluripotent stem cells, including octamer-binding protein 4 (Oct-4), Nanog, SRY-related HMG-box 2 (Sox-2), Lefty-A, Fibroblast growth factor 4 (FGF-4), reduced expression-1 (Rex-1), CFC-1, DPPA-3, and TDGF-1 [31, 37]. Oct-4 and Nanog are necessary for maintaining self-renewal and pluripotency of ESCs. Some AECs also express the surface receptor for stem cell factor (SCF) c-kit and Thy-1. Unlike ESCs, AECs do not synthesize telomerase that limit their long-term self-renewal. Therefore, they are not tumorigenic after transplantation. Whether AECs are clonogenic remains unclear. Under appropriate culture conditions, AECs exhibit the capability of differentiating into all three germ layers: endoderm (hepatic and pancreatic lineage), mesoderm (cardiac lineage), and ectoderm (neural lineage) [30, 31, 38].

AECs also may exhibit MSC-like phenotypes, which reflects in that AECs possess the potential of tri-mesodermal lineage differentiation potentials (adipogenic, osteogenic, and chondrogenic). They express cell surface markers like CD10, CD13, CD29, CD44, CD73, CD90, CD105, CD117, and CD166, some of which are specific to MSC. Besides, their surface is devoid of CD45, CD34, CD14, CD79 and human leukocyte antigen (HLA)-DR. Therefore, AECs may own similar benefits to MSCs, mainly immune-privilege and immunomodulatory effects [39, 40]. Human AECs expressing nonpolymorphic, nonclassical HLA-G and HLA-E. HLA-G is associated with the induction of immune tolerance [29, 41]. In addition, AECs do not express the costimulatory molecules CD80 (B7-1), CD86 (B7-2), CD40 or CD40 ligand (CD40L) in the presence or absence of interferon gamma (IFN-γ). They neither express the programmed cell death receptor 1 (PD1), nor its two ligands PD-L1 or PD-L2, which is also related to the induction of immune tolerance [42]. Akle et al. found that host immune rejection did not occur on the skin of volunteers after transplantation of immunotype-mismatched human AECs [43]. It is speculated that this phenomenon is due to the absence of polymorphic antigens HLA-A, -B, -C (major histocompatibility complex class I, MHC-I), -DR (MHC-II), and β2-microglobulin on the surface of human AECs [44]. Nevertheless, there are some conflicting reports on the immunogenicity of AECs. Fresh allogeneic AE expresses low levels of MHC class I antigens and sensitizes recipients when transplanted into mouse eyes, which can induce allospecific-delayed hypersensitivity but fails to acquire long-term memory. Although allogenic AE cannot arise intense immune rejection, they may not be completely immune-privileged tissue [41, 45].

Furthermore, AECs secrete a variety of immunoregulatory factors to exert adult stem-cell-like immunomodulatory effects [46]. They release a multitude of immunosuppressive molecules and growth factors, including interleukin (IL)-1 receptor antagonist protein, tissue inhibitors of metalloproteinase (TIMP) -1, -2, -3, and -4, IL-4, IL-10, transforming growth factor-β2 (TGF-β2), macrophage migration inhibitory factor (MIF), prostaglandin E2 (PGE2), insulin-like growth factor II (IGF-II), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [41, 47–50]. Collectively, these soluble cytokines can

modulate innate immunity and tolerance through inhibiting the activation of natural killer (NK) cells, suppressing the chemotactic activity of neutrophils, dendritic cells (DC) and macrophages, and promoting the polarization of macrophages phenotype transition from M1 to M2 [48, 51–53]. More importantly, not only do AECs modulate innate immunity, but work against adaptive immunity. AECs express the immunoinhibitory molecules Fas (CD59) and Fas ligand (FasL), which can regulate the complement immune system and induce apoptosis of T lymphocytes. AECs can inhibit the proliferation and activation of lymphocytes in a dose-dependent manner and induce the apoptosis of activated T and B cells. AECs also promote the polarization of T lymphocytes to regulatory T cells (Treg) and alter the relative proportion of different subsets of T cells including downregulating the ratio of Th1/Th17 [53–55]. Ultimately, AECs suppress allogeneic or other related immune responses.

Aside from the characteristics cited above, AECs are rich in lysosomes with large amount and high activity of lysosomal enzymes [32]. The high lysosomal activity in AECs is correlated with the active autophagy process occurring through placentation period [56]. AECs contain up to 17 lysosomal enzymes. Importantly, Scaggiante and colleagues have found that a considerable amount of lysosomal hydrolases can be released from AECs, such as sphingomyelinase, *N*-acetyl-β-glucosaminidase, α-fucosidase, β-glucuronidase, α-mannosidase, and arylsulfatase [57]. Recently, the therapeutic efficacy of AECs in lysosomal storage disorder (LSD)-murine models has been recognized [58]. These findings suggest the potential of using AECs as a source for cell replacement therapies for LSD patients.

Intriguingly, AECs also express other molecules involved in cell-cell interaction and cell adhesion, such as *E*-Cadherin, CD9, CD29, CD104, CD49e, CD49f, and CD44, which may facilitate cell migration and homing after transplantation [29, 59, 60].

Amniotic mesenchymal stem cells

At the hypoblast stage, extraembryonic mesoderm cells beneath the amnioblast eventually form the amniotic mesenchymal layer [25]. Cells from this layer are now termed amniotic mesenchymal stromal cells (AMSCs) by "International Placenta Stem Cell Society" [61]. AMSCs are spindle in shape, plastic-adherent, capable of producing fibroblast colony-forming units, and analogous to bone-marrow-derived MSCs and other adult sources. Human AM is thought to be a reservoir of MSC. It is estimated that an average of 5×10^8 AMSCs can be obtained from one AM [62]. A variety of protocols are used to isolate AMSCs from placenta. Different from the isolation of AECs by trypsinization, collagenase digestion is the most common protocol to dissociate AMSCs from the connective tissue. AMSCs are easily cultured and propagated in serum-free medium, with their augmented proliferative rates and high clonogenicity. It is estimated that approximately 2×10^6 cells can be obtained after culturing 1 g amniotic tissues for 3 days [63]. However, it is difficult to fully isolate a pure AMSC population without contamination of AECs [30]. Thus, initial studies demonstrated that AMSCs showed phenotypic characteristics of both mesenchymal and epithelial cells [10, 64].

Like AECs, AMSCs are multipotent and express stem cell markers Oct-4, Sox-2, Rex-1, Nanog, Nestin, SSEA-3, SSEA-4, c-kit, but not TRA1–61 [10, 64]. They have the multilineage differentiation potential and the ability to differentiate into multiple mesoderm-type cells such as osteocytes, adipocytes, and chondrocytes [38]. Similar to bone marrow and other adult tissue-derived MSCs, AMSCs show plastic adherence and fibroblast-like growth. Propagated AMSCs carry typical cell surface markers such as CD10, CD13, CD29, CD44, CD49d, CD49e, CD55, CD56, CD59, CD73, CD90, CD105, and CD166. However, they do not express hematopoietic stem cell surface markers including CD11b, CD14, CD34, CD45, CD117, CD119, and CD309 in vitro, which indicates hematopoietic cells are depleted in culture [29, 60, 61, 63]. They can be recognized by monoclonal antibody against stromal precursor cells-1. Like ESCs, AMSCs maintain long telomeres, which indicates a high expansion and proliferation rate in vivo and the capability of postponing senescence [38, 59]. AMSCs may be superior to adult MSCs in cell-based therapies since Alviano et al. observed that AMSCs present better proliferation and differentiation potential [64]. Apart from these features, AMSCs have a distinct expression pattern of the HOX gene family (e.g., HOX B2, B6, B7, B8).

The immunomodulatory activity and immune tolerance are the most prominent characteristics attributed to the current effective treatments by AMSCs. AMSCs show the immune-privileged profile with limited expression of HLA-A, -B, -DR [42]. Some inhibitory molecules or soluble factors of AMSCs were found to play significant roles in their immunosuppressive mechanisms. As AECs, AMSCs carry a high level of transcription factors and various soluble factors especially in the presence of IFN-γ, including IL-10, IL-1 receptor agonists (IL-1Ra), TGF-β, PGE2, and HGF [59]. But AMSCs are negative for the immunoglobulin-like transcript receptor (ILTR)-2, -3, and -4, which are expressed by T and B lymphocytes, NK cells, and DC [42]. In the presence of activated peripheral

blood mononuclear cells (PBMCs), AMSCs begin to synthesize the unstable immunosuppressive soluble factors indoleamine 2,3-dioxygenase (IDO), PGE2, and IL-10. AMSC-derived conditioned medium (CM) also possess broad immunomodulatory activities on various immune cells. Both AMSCs and their CM can restrain the proliferation of activated CD4 and CD8 T cells [53]. Pianta et al. substantiated that AMSCs could modulate T-cell subset. They observed a decrease in pro-inflammatory cytokines, including Th1-related (TNFα, IFNγ, IL-1β), Th2 (IL-5, IL-6), Th9 (IL-9), and Th17 (IL-17A, IL-22). Furthermore, CM generated from AMSCs significantly induced the Treg compartment, showing an induction of $CD4^+$ $FoxP3^+$ cells, and an augmentation of $CD25^+$ $FoxP3^+$ and $CD39^+$ $FoxP3^+$ Treg in the $CD4^+$ population [53, 65]. Except the soluble factors, AMSCs also express cell membrane bond molecules such as nonclassical class I HLA-G, PD-L1, B7 homolog 1 (B7-H1), and FasL [59]. Interestingly, the transwell assay result of physically separated AMSCs and PBMCs has suggested another inflammatory regulation mechanism by cell-to-cell contact [66]. Taken together, these immunomodulatory properties of AMSCs have made them attractive to treat certain diseases characterized by autoimmunity or inflammation.

AMSCs secret series of angiogenic factors to promote angiogenesis, including angiogenin, vascular endothelial growth factor (VEGF) and PDGF [13]. Studies had proved that AMSCs can promote angiogenesis through spontaneously differentiating into vessel-like structures and expressing endothelial specific genes and proteins in a mouse model of ischemic hindlimbs [67]. Capillary rarefaction in the kidneys predisposes to CKD progression. Therefore, the angiogenesis property makes AMSCs capable of promoting vascular regeneration or accelerating neovascularization after ischemic insult. Comparisons between AEC and AMSC are listed in Table 12.1.

TABLE 12.1 Comparison of AEC and AMSC.

	Markers	AEC	AMSC
Stemness and pluripotency (multipotency)	Oct-4	+	+
	Nanog	+	+
	Sox-2	+	+
	c-Myc	+	n.d.
	Rex-1	+	+
	Lefty-A	+	n.d.
	FGF-4	+	n.d.
	CFC-1	+	n.d.
	DPPA-3	+	n.d.
	TDGF-1	+	n.d.
	c-kit	+	+
	Thy-1	+	n.d.
	Nestin	n.d.	+
	SCF	n.d.	+
	SSEA-1	−	n.d.
	SSEA-3/4	+	+
	TRA1–60	+	−
	TRA1–81	+	−
Mesenchymal stem cell	CD10	+	+
	CD13	+	+
	CD55	n.d.	+
	CD73	+	+
	CD90	+	+
	CD105	+	+

Continued

TABLE 12.1 Comparison of AEC and AMSC—cont'd

	Markers	AEC	AMSC
Hematopoietic stem cell	CD11b	n.d.	–
	CD14	–	–
	CD19	n.d.	–
	CD34	–	–
	CD45	–	–
	CD79	–	n.d.
	CD117	+	–
	CD119	n.d.	–
	CD309	n.d.	–
MHC class	HLA-A/B (MHC class I)	± (low)	± (low)
	HLA-C (MHC class I)	± (low)	n.d.
	HLA-DR (MHC class II)	–	± (low)
	HLA-E	+	n.d.
	HLA-G	+	+
Lineage differentiation	Hematopoietic	n.d.	+
	Adipogenic	+	+
	Chondrogenic	+	+
	Osteogenic	+	+
	Neurogenic	+	+
	Myogenic	+	+
	Hepatic	+	+
	Cardiac	+	+
	Pancreatic	+	+
	Myogenic	+	+
Soluble cytokines	IL-4	+	n.d.
	IL-10	+	+
	TGF-β	+	+
	MIF	+	n.d.
	PGE2	+	+
	IGF	+	+
	HGF	+	+
	TRAIL	+	n.d.
	CD59 (Fas)	+	+
	FasL	+	+
	EGF	+	+
	bFGF	+	+
Angiogenesis	Angiogenin	+	+
	VEGF	+	+
	PDGF	+	+

TABLE 12.1 Comparison of AEC and AMSC—cont'd

	Markers	AEC	AMSC
Antiangiogenesis	IL-1 receptor agonist	+	+
	TIMP-1/2/3/4	+	n.d.
Cell-cell interaction	Integrin a 11	n.d.	+
	β1 integrins CD29	n.d.	+
	CD9	+	n.d.
	CD29	+	+
	CD44	+	+
	CD49d	+	+
	CD49e	+	+
	CD49f	+	n.d.
	CD54	n.d.	+
	CD104	+	n.d.
	CD166	+	+
	E-cadherin	+	+
Chemokines	CCL2	n.d.	+
	CXCL9	n.d.	+
	CXCL10	n.d.	+
	CXCL11	n.d.	+
Adhesion molecules	ICAM-1	n.d.	+
	VCAM-1	n.d.	+
	CD56	n.d.	+
Costimulatory molecules	CD40	–	n.d.
	CD40L	–	n.d.
	CD80 (B7-1)	–	+
	CD86 (B7-2)	–	n.d.
	PD1	–	n.d.
	PD-L1	–	+
	PD-L2	–	n.d.
Telomerase activity		–	n.d.
Tumorigenicity		–	–
Isolated measure		Trypsinization	Collagenase digestion

bFGF, basic fibroblast growth factor; *EGF*, epidermal growth factor; *FasL*, Fas ligand; *FGF-4*, fibroblast growth factor 4; *HGF*, hepatocyte growth factor; *HLA*, human leukocyte antigen; *ICAM-1*, intercellular adhesion molecule-1; *IGF*, insulin-like growth factor; *IL*, interleukin; *MHC*, major histocompatibility complex; *MIF*, macrophage migration inhibitory factor; *Oct-4*, octamer-binding protein 4; *PD1*, programmed cell death receptor 1; *PDGF*, platelet-derived growth factor; *PGE2*, prostaglandin E2; *SCF*, stem cell factor; *Rex-1*, reduced expression-1; *Sox-2*, SRY-related HMG-box 2; *SSEA-3/4*, stage-specific embryonic antigen-3/4; *TGF-β*, transforming growth factor-β; *TIMP*, tissue inhibitors of metalloproteinase; *TRA1-60/81*, tumor rejection antigen 1-60/81; *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand; *VCAM-1*, vascular cell adhesion molecule-1; *VEGF*, vascular endothelial growth factor; *n.d.*, not defined.

Regenerative medicine with amniotic stem cells

Regenerative medicine is the bold collection of biomedical approaches with the therapeutic purposes of restoring or replacing diseased and injured tissues or organs to resemble its original condition [68, 69]. Statistically, several thousands of people are acquiring a new lease of life by transplants per year over the world, and millions of people are benefiting from regenerative medicine [70]. There are two inseparable parts of regenerative medicine, stem cell therapy and tissue engineering. Stem cell therapies have been expected to bring promising benefits to patients suffering numerous disorders. Distinct from other stem cell types, the properties of ASCs make them unique. These cells own the features including lack of ethical concerns, ease of accessibility, richness in sources, few genetic mutations, multipotency and immunosuppression, which make them ideal candidates for allo- or xeno-transplantation and cell-based therapies [37].

Preclinical studies have shown that ASCs can alleviate lesions and promote repair in diseases from nearly every organ system of the body, including nervous system diseases [71, 72], diabetes mellitus (DM) [73], cardiovascular diseases [74], kidney regeneration (will be discussed in detail later), liver injury [75], lung injury [19, 76], ovarian failure [77], corneal wound [78, 79], and tumor [80]. Luo et al. found that human AECs transplantation reduced blood glucose level, increased plasma insulin content, and partially repaired the structure of islets in a streptozotocin (STZ) induced type I diabetes mouse model, suggesting AECs might be a promising cell-replacement therapeutic approach for this disease [81]. Tan and coworkers reported that human AEC-CM could mediate the functional repair after bleomycin-induced lung injury in mice by modulating the polarization, migration, and aggregation of macrophages [19]. Additionally, AMSCs have been the intriguing source of MSCs in cell transplantation and regenerative medicine. He et al. discovered similar therapeutic effects of AMSCs on pulmonary fibrosis rats to MSCs from other sources. A significant elevation in survival rate with the decreased infiltration of $CD3^+$ T cell and reduced expression of inflammatory cytokines were shown after AMSCs injection [76].

In the field of tissue engineering, both AECs and AMSCs are found effective in some conditions with their exceptional mechanical and biochemical properties, such as dermatological defects, neurosurgery, ophthalmic surgery, and vagina surgery [82]. ASCs are now used to develop an artificial tissue-engineered biomaterials [13]. For example, Jiang et al. used AECs and fibroblasts to form an organotypic skin, which could exhibit the main ultrastructural features like normal skin [83]. Another study cultured AECs on the AMSCs seeded fibrin scaffold to develop a bilayer tissue-engineered skin for skin transplantation. They concluded that bilayer tissue-engineered skin remarkably accelerated wound healing as a result of various ECM components secretion [84].

Clinically, ASCs have been used in some trails. The earliest clinical trial of AECs could be traced back to 1981. Isolated human AECs were transplanted into volunteers' skin, and no adverse effects especially tumor formation occurred [43]. In the next decade, nontumorigenesis effects of AECs had been repeatedly proved by other researchers [85–87]. Despite carrying long telomeres, AMSCs do not form teratomas in vivo either [88]. These data indicate that even with the stemness of ASCs, they are distinguished from ESCs with genetic stability, suggesting ASCs may be one of the safest stem cell sources for clinical cell-based therapy in regenerative medicine. Given the strong preclinical data, ASCs by now have been brought into clinical use, which will be described in detail in the following part.

Taken together, research on ASCs have switched from the cellular origins, phenotypes, isolation, culture, and characterization to preclinical and clinical applications during the past few decades. In order to tap into the therapeutic potential of ASCs, the focus of these cells has now shifted more to storage and banking protocols, facilities, manufacturing techniques, and the development of intellectual property to realize commercialization. In 2009, the first private stem cell banking for ASCs was opened by Biocell Center (www.biocellcenter.com) [70]. The prospect of banking ASCs for future use in autologous cell therapies represents an important advance in clinical application.

Amniotic stem cells ameliorate renal injury and accelerate renal repair

To date, a few proof-of-concept applications of ASCs for renal injury have been reported. However, as already described earlier, ASCs possess many outstanding features and functions worthwhile exploiting in this setting.

AKI is a clinical syndrome with high morbidity, high mortality, and high medical expenditures, its pathogenesis is complex and the existing treatments are limited [89]. Studies have shown that AKI can generate a large number of pro-inflammatory factors such as IL-2, -6, -8, -18, NF-κB, and so forth, causing a series of immune system

dysfunctions, subsequently leading to vascular endothelial cell dysfunction, glomerular and tubular cells apoptosis and necrosis, and eventually aggravated kidney damage [90, 91]. Liu et al. cocultured AECs with $CD4^+$ T cells sorted from AKI patient-derived PBMCs and found that AECs could effectively inhibit the proliferation of $CD4^+$ T cells and reduce the expression and release of IL-2 [92]. The high level of IL-2 in peripheral blood might be associated with the early onset of AKI. Therefore, AECs may reduce AKI by inhibiting the inflammatory response. Our lab established ischemia-reperfusion injury induced AKI (IRI-AKI) mouse model with different severity of renal injury to explore the role of AECs on IRI-AKI. We found that systematically administrated AECs could reduce mortality and improve the systemic status of AKI mice. AECs ameliorated renal function by reducing apoptotic cells, lessening peritubular capillary loss and alleviating kidney local immune response. The antiinflammatory action was associated with the significantly decreased expression of inflammatory cytokines (IL-8, IFN-γ, and TNF-α) and reduced infiltration of T and B lymphocytes and neutrophils [93].

CKD is a gradual loss of kidney function, which can cause irreversible glomerular and tubular damage, eventually leading to kidney failure and other organ damage [94]. Dialysis and kidney transplantation are currently two effective treatments for CKD, but the former cannot fully restore kidney function, while transplantation also faces a major problem of donor shortage. Emerging cell therapies have brought new hope to patients with CKD. It has been shown that stem cells derived from placenta can both improve and delay the progression of renal fibrosis in animal models of CKD [95, 96]. Cetinkaya and colleagues constituted a rat model of CKD by applying aristolochic acid (AA), and investigated whether AMSCs could repair fibrosis. Compared with AA group, there was a decreased accumulation of collagen and expression of IL-6 in AMSCs injected group, indicating an amelioration of tubulointerstitial fibrosis. Besides, augmented PCNA and Ki-67, as well as decreased PARP-1 and p57 expression in AMSCs group, indicated the promotion of proliferation and the inhibition of apoptosis [95]. Another research was applied in domestic cat, a type of animal that is capable of naturally developing CKD. After feline AMSCs injection, significant improvement in renal function was observed involving decrease in serum creatinine and urine protein concentrations, and augmentation of urine specific gravity. Nevertheless, the kidney architecture and morphology did not change [96]. In our study, a reduction of renal fibrosis was shown after AEC treatment in IRI-AKI mice, which means AECs may stabilize the disease condition and delay the progression from AKI to CKD [93].

Whether ASCs will be a better candidate for cellular therapies in kidney disease still needs to be precisely investigated in different AKI or CKD animal models; however, existing evidence has already highlighted the effective therapeutic potential.

Homing and differentiation of exogenous amniotic stem cells

Cell migration, homing and differentiation are one of the major mechanisms of action for cell-based therapy. Mounting experiments have confirmed that AECs can differentiate into many cell types in vitro, but whether its role in vivo is achieved by homing to injured tissue and differentiation still needs further investigation.

To figure this out, scientists have used different administration routes to explore the homing and differentiation of exogenous ASCs. Srinivasan et al. employed two different routes of administration to explore the efficacy of delivering AECs to liver. They found that cells infused through the tail vein would localize in multiple organs, mainly accumulated in lung and would be cleared in a short time. Contrarily, almost all cells were restricted to the site of infusion and homed to the liver after splenic injection and could be retained in the body for a long time. These results demonstrated that portal vein injection via spleen was an effective delivery of AECs targeting the liver [97]. Luo et al. further observed that only a small amount of AECs could home to the damaged organs. Dismayingly, the homing cells cannot differentiate into homologous cells, suggesting the therapeutic effect of AECs might not be achieved by homing, as well as differentiation [81]. Zhang and colleagues also proposed that it was with small probability that the functional recovery of injured ovaries by AECs was through transdifferentiating into ovarian granulosa cells [98].

As one cell type of MSCs, AMSCs may exert analogous modes of action as MSCs. It has been proposed that additional benefits of the reduction in infiltrating inflammatory cells may be achieved when MSCs homed to spleen. In general, inflammation may promote AMSCs migrating and homing to the injured site [59]. Matrix metalloproteases (MMPs), chemokine receptors, and chemokine receptor type 4 (CXCR4) are essential for AMSCs to migrate toward injured sites. After migration, resident AMSCs may secrete chemokines (CCL2, CXCL9, CXCL10, and CXCL11) and express cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 to alleviate injury by attracting immune cells contacting with them at inflammation site [29]. In fact, either

distal or local homing of MSCs in diverse kidney disease models was rarely investigated in detail despite the treatment effect was obvious. Cetinkaya et al. observed the localization of AMSCs in kidney tissues after injecting cells into tail vein of rats by immunohistochemistry analysis, but whether AMSCs differentiated into renal cells was not mentioned [95]. The route of AMSCs delivery is important as well. Scientists concluded that intravenous infusion for AMSCs delivery was more efficacious than intrarenal route in feline spontaneous CKD [96]. However, stem cells, particularly MSCs, often tend to accumulate in filtering organs such as lung, spleen, or liver when they are systemically administered via intravenous infusion. Even worse, MSCs may get trapped in the lung as microemboli, endangering life [99].

As the multilineage differentiation potential in vitro, ASCs are expected to differentiate into some renal parenchymal cells under certain conditions in vivo. However, despite a positive therapeutic signal, our own data showed very low kidney tissue integration of AECs from the beginning of injection till 7 days postinjection in the IRI-AKI mice [93]. Elucidating the precise mechanism whether AECs or AMSCs migrating to the injured kidney may have great consequences for human therapy and that effective delivery of these cells to damaged organ matter equally.

Paracrine and endocrine mechanism of exogenous amniotic stem cells

Accumulating evidence favors the paracrine and/or endocrine mechanism of ASCs that exerts the therapeutic effects in kidney diseases. Rather than direct incorporation into renal components, AECs and AMSCs can secrete soluble factors and renotropic factors, such as cytokines, growth factors, chemokines, microvesicles/exosomes, and cell adhesion molecules [66, 99]. These secretomes can promote cell proliferation, migration, dedifferentiation, and angiogenesis, and attenuate renal apoptosis, inflammation, and fibrosis, thereby facilitates kidney regeneration [29, 59].

Immune components play a critical role in triggering tissue regeneration and injury [100]. Given the importance of inflammation in the pathophysiology of AKI [90], exploring the exact immunomodulation mechanism of ASCs may contribute to better understanding of their role in renoprotection. ASCs are immunologically tolerant and allogeneic AECs or AMSCs do not induce a proliferative T-cell response. ASCs express limited levels of MHC I and II molecules and lack major costimulatory molecules, among others [42, 44]. Most interestingly, they constitutively express ILTR-recognized HLA-G and abrogate activating signals received from T and B cells, as well as DC and NK cells [29]. Growing studies in vivo and in vitro have reported that epithelial and mesenchymal cells from the AM possess extensive immunomodulatory activities on different immune cell subpopulations of both innate (monocytes, macrophages, DC, neutrophils, and NK cells) and adaptive (T and B lymphocytes) immunity [29, 53]. Both AECs and AMSCs can suppress T-cell proliferation in a dose-dependent manner with proper stimuli. Intriguingly, the CM generated from cultured AECs and AMSCs also shows the antiproliferative merits on lymphocytes, supporting the involvement of secreted factors [53, 101]. ASCs possess the potential of minimizing the expression of pro-inflammatory Th1-related cytokines IFN-γ and TNF-α. Specifically, AMSCs and their CM reduce the expression of Th9 (IL-9) and Th17 (IL-17A, IL-22) cytokines while augmenting IL-2 and TGF-β expression. Most importantly, AMSCs and their CM suppress the proliferation of both activated $CD4^+$ and $CD8^+$ T cells [65]. T cells exert essential roles in immune-related and ischemic kidney disease, thus the merit of ASCs to modulate T-cell response is probable to contribute to the therapeutic options in AKI. In compliance with this notion, Liu et al. showed that AECs inhibited the proliferation of $CD4^+$ T-cell derived from PBMC in patients with AKI by inhibiting the expression and release of IL-2 [92]. Vidane et al. showed a decrease in lymphocyte count after the second administration of AMSCs along with the significant improvement of clinical parameters in renal function. They speculated that the renoprotective effect of AMSCs could be realized by intense paracrine activity in cats with CKD. AMSCs had a long-term action and thereby stabilized the disease [96].

In addition, ASCs release high level of IL-10 and IL-1Ra, which counteracts inflammatory cytokines (TNF-α, IL-1, IL-8, IL-6) to inhibit their production from immune cells [102]. AMSCs can suppress proliferation of T cells via autosecreting IL-10 or inducing other immune cells to produce IL-10 by molecules like IDO, and then interfere with the growth and activity of T lymphocytes and NK cells [59]. Cetinkaya and coworkers investigated an antifibrotic profile of AMSCs with antiinflammatory (mainly the reduction of IL-6 expression), antiproliferative, and antiapoptotic mechanism in murine CKD model [95]. TGF-β is secreted to extracellular matrix (ECM) as an inactive latent form. After activation, TGF-β interacts with its receptor on immune cells and triggers exactly opposite effects of antiinflammation or pro-inflammation in a context-dependent manner. AM-derived cells secrete TGF-β to inhibit the inflammatory cytokine production by classical macrophages (M1) and promote the activation of alternative macrophage (M2) to secret antiinflammatory cytokines [59, 103, 104]. The increased secretion of TGF-β also corroborates the induction of Treg cells. Tan et al. used a mouse model of bleomycin-induced lung fibrosis and found the ability of AECs in inducing FoxP3 transcription in murine CD4 naïve cells by promoting

TGF-β secretion [54]. On the contrary, Alcaraz et al. had shown that cultured AECs undergo epithelial to mesenchymal transition (EMT) through autocrine of TGF-β [105]. TGF-β has been proposed the main link of renal fibrosis. PGE2 is a dominant immunomodulatory mediator secreted from ASCs that carries various immunosuppressive effects. This molecule suppresses immune response by inducing the production of cAMP to inhibit T-cell activation and reduce inflammatory cell migration by regulating chemokine production [106].

Macrophages are highly heterogeneous and plastic innate immune cells. They play a versatile role in the maintenance of kidney homeostasis and the imbalance of M1/M2 macrophage phenotypes contributes to fibrosis or tissue repair after AKI [104]. It is reported that AMSCs and AECs can block the maturation and differentiation of monocytes into pro-inflammatory M1 macrophages by reducing LPS stimulated TNF-α and IL-6 production. Moreover, the monocytes exposed to AMSC and their CM express M2 markers, including CD14, CD23, CD163, and CD209. M2 macrophage possesses higher phagocytic activity, generates higher level of IL-10 and lower level of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-8, IL-12, TNF-α, MIP-1α, MIP-1β, MIG, RANTES, and IP-10, which may contribute to the resolution of injury [52]. Similarly, we observed an increased percentage of macrophages after IRI with a prominent M2 phenotype implying that AECs could alleviate IRI-AKI through promoting M2 macrophage polarization. Other potential immunosuppressive actions of ASCs include promoting apoptosis or inhibiting proliferation of B cells, preventing the activation, cytotoxicity and migration of DC, NK cells, and neutrophils [53]. These mechanisms of ASCs are summarized in Fig. 12.2.

Besides immunosuppressive factors, ASCs secrete a broad array of growth factors, trophic factors, and angiogenic factors, including HGF, PDGF, TGF-β, VEGF, epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), IGF-1, and basic fibroblast growth factor (bFGF) [107]. Several factors facilitate cell migration, some enhance epithelial proliferation, and some promote vessel formation or maturation [108]. For example, IGF-1 and HGF are epithelial mitogens and morphogens that could protect mice against ischemic kidney injury [109, 110]. Some proteins involved in EGF, PDGF, and FGF signaling pathways are secreted by AECs with antifibrotic properties [13]. HLA-G secreted by AECs exerts an effective antifibrotic action through inhibiting the production of collagen in fibroblasts induced by TGF-β1 via the TGF-β1/Smad3-dependent pathway [111]. Zhang et al. confirmed the secretion of TGF-β1, GDF9, and BMP15 from AECs in a paracrine manner. They observed the activation of the TGF-β/Smad pathway and inhibition of chemotherapy-induced apoptosis and fibrosis, and promotion of the angiogenesis in damaged sites [98]. AMSCs also express mediators such as FGF1, GRO-α, SDF-1 to promote angiogenesis, specifically [112]. All these merits make them an alternative candidate for therapeutic efficacy in renal injury. Taken together, ASC-derived paracrine effects may render these cells a promising strategy for the reactivation of repair or regeneration in renal disease, which needs to be further elucidated.

Amniotic stem cell-derived exosomes and renal injury

Introduction of exosomes

In the stem cell research arena, growing interest has focused on the ASC-secreted EVs to be potential carriers of paracrine signals. The classification of EVs generally falls into two major subsets according to their size and origin, ectosomes and exosomes [113]. Ectosomes are vesicles generated via outward budding of the plasma membrane, which include three kinds of vesicles named microvesicles, microparticles, and large vesicles in the size range between 50 nm and 1 μm in diameter. Exosomes are EVs of endosomal origin with a size range of 40–160 nm in diameter (approximately 100 nm on average).

In general, exosomes are generated by a continuous process [114–116]: Firstly, plasma membrane envelops cell-surface proteins, soluble proteins associated with extracellular milieu and invaginates to form a cup-shaped structure. This process is the formation of an early-sorting endosome (ESEs). Then, ESEs further mature into late-sorting endosomes (LSEs). Thirdly, LSEs ultimately generate a lot of multivesicular endosomes, known as multivesicular bodies (MVBs), by a second invagination of the endosomal limiting membrane. Meanwhile, the future exosomes, termed intraluminal vesicles (ILVs) at this step, become enwrapped within the MVBs. The fusion of MVBs with plasma membrane and exocytose results ILVs released as exosomes, eventually [15].

Exosomes contain a multitude of constituents such as DNA, microRNA, lipids, cell-surface proteins, cytosolic proteins, nucleic acids, amino acids, and metabolites [15]. Therefore, they can perform a wide range of biological functions. In various diseases, exosomes not only possess therapeutic potential but also have the capability to aid in disease diagnoses. Numerous preliminary reports conferred the antioxidation, antiinflammatory, antifibrosis, and proproliferation effects of ASC-derived exosomes in various conditions, including premature ovarian insufficiency

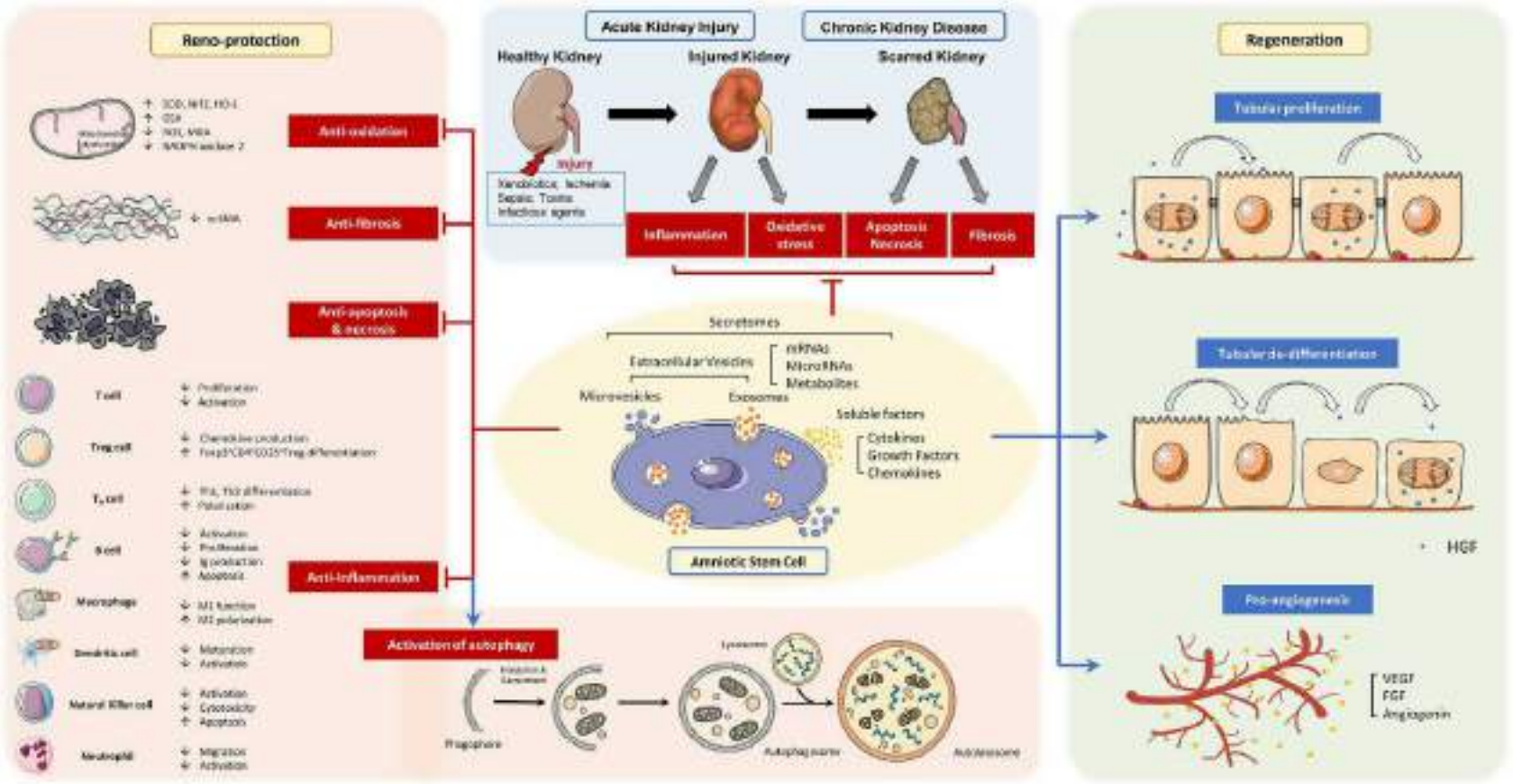

FIG. 12.2 Possible mechanism (effects) of amniotic stem cells in renal injury. Various types of insults (ischemia, toxins) instigate acute renal injury that results in renal inflammation and tubular damage. The renal damage subsequently increases the production of pro-inflammatory cytokines and chemokines in renal cells and thereby inducing oxidative stress. Sustained oxidative stress can exacerbates renal damage that partly reflected by augmented cell apoptosis and necrosis. Severe damage together with continually expressed pathogenic factors lead to progressive kidney injury and numerous profibrotic mediators can be released in response to the persistent inflammation. These profibrotic factors drive myofibroblast activation and accumulation, thereby promoting renal fibrosis. The pathophysiological process involved in the acute and the chronic stages could be blocked by ASC actions via diverse mechanisms. ASCs release a multitude of cytokines, growth factors, and microvesicles that can exert a series of renoprotective and regenerative actions on injured kidney tissues through various paracrine mechanisms: proliferation and differentiation, antioxidative stress, proangiogenesis, antifibrosis and antiapoptosis, immunomodulation and immunosuppression, and activation of autophagy. *Blue arrow*: enhancement; *Red T-bar*: reduction.

[117], wound healing [118], idiopathic pulmonary fibrosis [19]. Deductively, exosomes derived from ASCs have emerged as new mediators of cell-to-cell communication that may induce renal cell reprogramming and tissue regeneration via transferring proteins, functional mRNAs, and miRNAs.

Amniotic epithelial cell-derived exosomes and acute kidney injury

AEC-derived exosomes (AEC-Exos) have both antiinflammatory and antifibrotic properties. On the one hand, AEC-Exos exert antiinflammatory effects by reducing neutrophil myeloperoxidase activity, inhibiting the proliferation of CD3/CD28 activated T cell, enhancing phagocytic activity of macrophages, as well as changing their polarization state [19, 119]. On the other hand, AEC-Exos are rich in numerous microRNAs (miRNA). miRNA is a special class of endogenous, highly conserved noncoding single-stranded RNA with a length of 21–23 base pairs. miRNA is widely distributed in single-cell and multicell organisms, does not have open reading frame (ORF) and cannot be translated into proteins. Functionally, miRNAs bind to the specific sites to participate in the proliferation, differentiation and apoptosis of cells, as well as many physiological processes via silencing the expression of target genes [120–122]. miRNAs carried by AEC-Exos may contribute to their antifibrotic characteristics [19]. Among them, miR-27a and miR-34a inhibit fibrosis by affecting the differentiation and proliferation of myofibroblasts [123, 124]; miR-150 and miR-194 reduce the collagen deposition from fibroblasts by targeting c-MYB and RAC1 expression [125]; miR-23a and miR-203a can inhibit the expression of Smad2 and Smad3 in the TGF-β signaling pathway, respectively, thereby inhibiting EMT [121]. In vitro experiments showed that AECs secreted a high level of miR-101, which contributed to the suppression inflammatory response in AKI patients through preventing the formation of a transcription factor complex between the target gene *c-Rel* and NF-κB in the nucleus [92]. Thus, these intriguing findings show that exosomes-mediated transfer of miRNA is an additional and potential key mechanism by which ASCs exert renoprotective effects.

Our previous study demonstrated that exosomes derived from AECs were as effective as AECs themselves in ameliorating injury and promoting survival in severe IRI-AKI mice model. To figure out specific proteins carried by AEC-Exos, a comprehensive analysis of the proteome had been carried out. Predictably, a large proportion of exosome proteins was annotated as either of extracellular or membrane origin, with molecular functions of protein binding, integrin binding, as well as ECM structural constituent. Biological processes including "extracellular matrix organization," "cell adhesion," "leukocyte migration," as well as "angiogenesis" and "positive regulation of cell proliferation," were enriched in AEC-Exos. A series of proteins or genes were involved in the IGF signaling (IGFBP2, IGF2), HGF signaling (HGFAC), TGFβ signaling (TGFBI, LTBP1, BMP1), and Wnt signaling (DKK3, SFRP1, LRP1) [93].

Amniotic mesenchymal stem cell-derived exosomes and kidney injury

There are few published studies on AMSCs in kidney injury. Gao et al. found that the miRNAs (miR-135a) produced from AMSC-derived exosomes could promote cell proliferation and migration by targeting corresponding mRNAs, thereby accelerating wound healing and repair in rats [20]. Another in vitro experiment also confirmed that AMSC-derived exosomes promoted fibroblast migration through their miR-135a [126]. A miRNA-target network analysis revealed that exosomes-derived miRNAs of AMSCs governed T-cell survival/proliferation and immune response, which may interfere with the paracrine immunomodulatory effects [66]. Abnormal inflammatory cell infiltration is one of the causes of acute and chronic kidney injury. Given the unique immunosuppressive effects of AMSCs, we have reasons to believe AMSCs may repair kidney damage and improve renal function by reducing inflammation.

Clinical trials for amniotic stem cells in renal injury

As early as 1995, AM transplantation had been successfully performed to reconstruct ocular surfaces in patients [37]. Growing evidence has shown the enormous potential of ASCs. So far, several clinical trials are being recruited and are in progress. As of April 2020, the NIH Clinical Trial (NCT; www.clinicaltrials.gov) and Australian New Zealand Clinical Trials Registry (ANZCTR; www.anzctr.org.au) for clinical trials list over 30 trails with a search term "amniotic epithelial cell" or "amniotic mesenchymal stem cell" (AEC: 20 trials in NCT and 5 trials in ANZCTR; AMSC: 5 trials in NCT). After excluding trials that utilize the AM as a scaffold and some others, there are 17 clinical trials (AEC: 12 trials in NCT and 4 trial in ANZCTR; AMSC: 1 trial in NCT) listed for using ASCs as a biological intervention (Table 12.2).

TABLE 12.2 Clinical trials using amniotic stem cells.

	Registration number	Title	Status	Conditions	Interventions	Phase	Study start	Locations
1	NCT03107975	Effect of human amniotic epithelial cells on children with spastic cerebral palsy	Unknown	Spastic cerebral palsy	Human amniotic epithelial cells	I	April 1, 2017	The second affiliated hospital of Fujian Medical University, Quanzhou, Fujian, China
2	NCT03031509	Treatment of nonunion of limb fracture with human amniotic epithelial cells	Not yet recruiting	Nonunion fracture	Human amniotic epithelial cells	I & II	December, 2017	Shanghai East Hospital, Shanghai, China
3	NCT00344708	Transplantation of tissue cultured human amniotic epithelial cells onto damaged ocular surfaces	Withdrawn	Corneal epithelial dystrophy	Human amniotic epithelial cells	Not applicable	September, 2000	University of Texas Southwestern Medical Center, Dallas, Texas, The United States
4	NCT02912104	A therapeutic trial of human amniotic epithelial cells transplantation for primary ovarian insufficiency patients	Unknown	Primary ovarian insufficiency	Human amniotic epithelial cells	I	September, 2016	The International Peace Maternity and Child Health Hospital, Shanghai, China
5	NCT02959333	Human amniotic epithelial cells for treatment of bronchial fistula	Unknown	Bronchial fistula	Human amniotic epithelial cells	I	October, 2016	The second affiliated Hospital of Fujian Medical University, Quanzhou, Fujian, China
6	NCT03764228	Human amniotic epithelial cells prevent acute graft-versus-host disease after hematopoietic stem cell transplantation	Not yet recruiting	Acute-graft-versus-host disease	Human amniotic epithelial cells	Not applicable	April 1, 2020	Peking University People's Hospital, Beijing, China
7	NCT03207412	Human amniotic epithelial cells treatment for ovarian insufficiency	Unknown	Premature ovarian failure	Human amniotic epithelial cells	Not applicable	December 22, 2017	The second affiliated hospital of Chongqing Medical University, Chongqing, China
8	NCT03223454	Human amniotic epithelial cells for Ashermaṅs syndrome	Not yet recruiting	Ashermaṅs syndrome	Human amniotic epithelial cells	I	October, 2017	The second affiliated Hospital of Chongqing Medical University, Chongqing, China
9	NCT03381807	Human amniotic epithelial cell in treatment of refractory severe i ntrauterine adhesion	Not yet recruiting	Intrauterine adhesion	Human amniotic epithelial cells	Early phase I	March 1, 2018	The second affiliated hospital of Medical College of Zhejiang University, Hangzhou, Zhejiang, China
10	NCT02961712	Immunotherapy of natural killer (NK) cells in human t lymphotropic virus type 1 (HTLV-1) associated myelopathy	Unknown	HTLV-1 associated myelopathy (HAM)	Human amniotic epithelial cells	I	July, 2016	The second affiliated hospital of Fujian Medical University, Quanzhou, Fujian, China

11	NCT03759899	Haecs are preliminarily applied in allogeneic hematopoietic stem cell transplantation	Not yet recruiting	Leukemia	Human amniotic epithelial cells	Not applicable	April 1, 2020	Peking University Institute of Hematology, Beijing, China
12	NCT02649621	The improvement of limbal stem cell deficiency (LSCD) in unilateral stem cell damage by amniotic membrane extract eye drop (AMEED)	Completed	Limbal stem cell deficiency (LSCD)	Amniotic membrane extract eye drop (AMEED)	I	August, 2011	Royan Institute, Tehran, Iran
13	ACTRN12614000174684	A pilot study evaluating the safety of intravenously administered human amnion epithelial cells for treatment of bronchopulmonary dysplasia (BPD) in premature babies	Recruiting	Bronchopulmonary dysplasia	Human amniotic epithelial cells	I	December, 2014	Monash Institute of Medical Research, Melbourne, Victoria, Australia
14	ACTRN12618000920291	Human amnion epithelial cells for prevention of bronchopulmonary dysplasia: a phase 1 dose escalation study	Recruiting	Bronchopulmonary dysplasia	Human amniotic epithelial cells	I	May, 2018	The Royal Womeńs Hospital, Melbourne, Victoria, Australia
15	ACTRN12616000437460	A pilot study evaluating the safety of intravenously administered human amnion epithelial cells for the treatment of hepatic fibrosis	Recruiting	Liver fibrosis	Human amniotic epithelial cells	I	April, 2016	Monash Medical Centre, Melbourne, Victoria, Australia
16	ACTRN12618000076279	I-ACT: Phase I trial of amnion cell therapy for ischemic stroke to establish the maximum tolerable dose	Recruiting	Ischemic stroke	Human amniotic epithelial cells	I	January, 2018	Monash Medical Centre, Melbourne, Victoria, Australia
17	NCT04318600	Allogeneic amniotic mesenchymal stem cell therapy for lupus nephritis	Completed	Lupus nephritis	Human amniotic mesenchymal stem cells	I	January 1, 2014	Yańan affiliated hospital of Kunming Medical University, Kunming, Yunnan, China

In the human trials reported presently, ASCs are mainly tested in the phase I and II clinical trials and revealed a very good safety record. Several trials have acquired positive results in a multitude of human diseases including bronchopulmonary dysplasia (BPD) [127], hepatic fibrosis [128], ischemic stroke [129], graft-versus-host disease, and many others. The world's first-in-human clinical trial using AECs for six premature babies with BPD was recently completed in Australia (trial ID: ACTRN12614000174684). This trial evaluated and proved the safety of AECs with no acute adverse events such as anaphylaxis, infection, rejection, or teratomas formation during and after cell administration in any of infants [127]. ASCs are being investigated in several kidney diseases, particularly those in which inflammation exerts a prominent role. Two clinical trials of kidney disease proposed application of ASCs have been approved in China. The trial entitled "Allogeneic amniotic mesenchymal stem cell therapy for lupus nephritis (LN)" is currently underway for evaluating the safety, feasibility and preliminary efficacy of human AMSCs for the treatment of LN (trial ID: NCT04318600). Despite the recruitment phase has been completed, the preliminary results of this trial have not been reported. Zeng et al. used AECs to treat a patient with membranous nephropathy (MN) after allogeneic hematopoietic stem cell transplantation in blastic transformation of chronic myeloid leukemia. After 4 weeks of AECs infusion, kidney function of the patient had been significantly improved, reflected by the decrease of 24-h urine protein and urine β2 microglobulin, and the elevation of serum albumin. Inspiringly, the serum albumin level of the patient had returned to normal after 6 weeks of infusions and the nephrotic syndrome (NS) was cured after 11 months of AECs administration [130].

Although ASC therapies are a promising field and several small phase I and phase II trials have proved modest improvements in both physiological and biochemical parameters, there are still challenges remained in the application of clinical-grade stem cells: (1) microembolic phenomenon caused by improper cell infusion method and excessive cell concentration; (2) scarce and unavailable long-term follow-up information; (3) lack of official certification for its safety and effectiveness and some others. Although there have been clinical applications of ASCs, the safety and efficacy of ASCs should be further examined by clinical trials conducted with the highest scientific and ethical standards.

Conclusions

Stem cells are critical to maintaining cellular homeostasis and restoring injured tissues and organs. Evidence supporting the beneficial effects of ASCs in regenerative medicine has increased exponentially in the past decades. On the one hand, ASCs possess a broad differentiation potential to produce the entire spectrum of mature progeny including renal cells and mobilize endogenous regenerative capacity of kidney. On the other hand, given the robust evidence favoring a paracrine mechanism of treatment with ASCs, more work needs to identify the secreted soluble factors and the role of secreted vesicles like exosomes that mediate paracrine actions. Though much remains to be learned regarding feasibility and safety of ASCs in the clinical setting, this cell population holds great promise to treat renal disorders and will eventually be successfully used in regenerative medicine for kidney diseases.

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CHAPTER

13

Regenerative potential of stem-cell-derived extracellular vesicles

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Introduction

Extracellular vesicles (EVs) are nanometer-sized vesicles, which mediate cell-to-cell communications by transferring DNAs, mRNAs, microRNAs, noncoding RNAs, as well as proteins to recipient cells [1]. Substantial progress has been made in our understanding of the biogenesis, biological properties, and their role in diseases. These endogenous vesicles show low immunogenicity and their therapeutic functions are similar to the cells of origin [2]. In particular, EVs derived from stem cells are good candidates for promoting regeneration and repair of injured tissues [3]. The present chapter summarizes current knowledge about regenerative potentials of stem-cell-derived EVs in preclinical models of kidney diseases.

Classification of EVs and their biogenesis

Extracellular vesicles (EVs) are heterogeneous populations of membranous vesicles released by cells in an evolutionarily conserved manner. These vesicles are categorized into two broad subfamilies based upon their size, subcellular origin, and the mechanisms responsible for their generation (Fig. 13.1). One major subfamily is comprised of relatively "small" vesicles, typically with a diameter ranging from 40 to 100 nm. These small membranous vesicles are generated by series of processes beginning with intracellular invagination of clathrin-coated microdomains of the plasma membrane. Multiple pathways, including the endosomal sorting complexes for transport (ESCRT) protein machinery, are involved in formation of early endosomes, intraluminal vesicles, and multivesicular bodies (MVBs). At this stage, those MVBs destined for degradation are trafficked to lysosome ("degradative MVBs"), but some MVBs fuse with the plasma membrane and are subsequently released into extracellular environment that are now referred to as exosomes [4, 5]. The ESCRT family comprises four protein complexes (ESCRT-0, -I, -II, and -III) and accessory proteins (Alix, VPS4, and VTA-1) that function to sequentially bind future exosome cargos and form intraluminal vesicles by incorporating these cargos. ESCRT-III complex forms spirals that induce the inward budding and fission of vesicles to form MVBs. Meanwhile, ESCRT-independent mechanisms of exosome biogenesis and release have also been identified [6–8]. Pathways involving neutral sphingomyelinases (nSMases) that generate the bioactive lipid ceramide [9] and resulting increases in ceramide levels can promote different aspects of MVB maturation.

The other subfamily is comprised of relatively "large" vesicles, typically in range of 100–1000 nm diameter. These large vesicles are usually called "microvesicles (MVs)" and arise from outward budding of the plasma membrane

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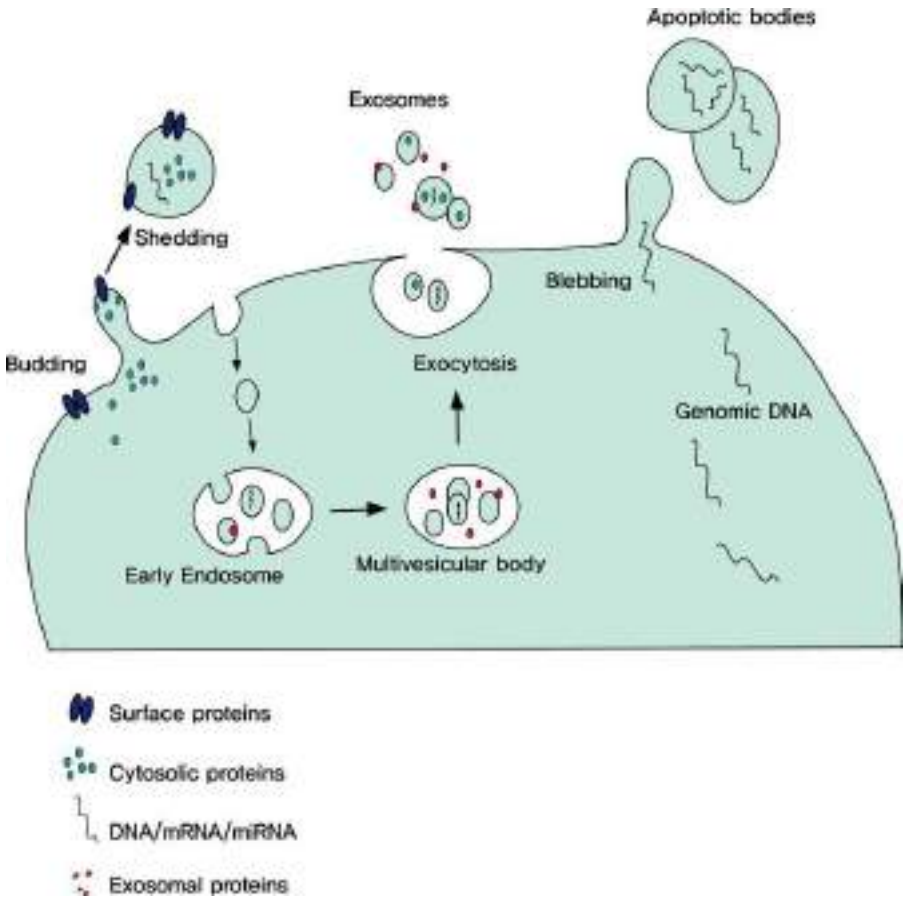

FIG. 13.1 Schematic representation of the mechanisms of extracellular vesicles formation. Microvesicles (0.2–2.0 μm) are formed via budding and shedding from the plasma membrane of cells and therefore may contain specific surface markers from the cell of origin. In contrast, exosomes (50–100 nm) are formed intracellularly through a sorting pathway involving intermediate organelles such as the early endosome and a late multivesicular body, which fuses with the plasma membrane to release exosomes via exocytosis.

through dynamic redistribution of phospholipids. Rearrangement of cytoskeletal actin and myosin that is regulated by Ras-related GTPase ADP-ribosylation factor 6 (ARF6) is implicated in formation of MVs [10]. MV production is observed in a variety of cells in a resting state, but the production can be significantly elevated under various stimulations, including cellular stress (e.g., DNA damage), and cytokine exposure [1, 11–15], and increased intracellular calcium concentrations with resulting activation of several key enzymes (e.g., calpain, scramblase, and floppase). However, more recent studies have shown involvement of ESCRT machinery, as well as other auxiliary proteins (ALIX, TSG101, and VPS4) in formation of MVs [16].

Schematic representation of the mechanisms of formation of extracellular vesicles. Microvesicles (0.2–2.0 μm) are formed via budding and shedding from the plasma membrane of cells and therefore may contain specific surface markers from the cell of origin. In contrast, exosomes (50–100 nm) are formed intracellularly through a sorting pathway involving intermediate organelles such as the early endosome and a late multivesicular body, which fuses with the plasma membrane to release exosomes via exocytosis.

Contents of EVs and their mechanism of action

EVs contain endogenous substances that vary with respect to the originating cell type, biogenesis mechanisms, as well as physiologic or pathologic conditions. These endogenous substances encompass a broad spectrum of proteins, lipids, and nucleic acids. Proteins that are commonly found in EVs include proteins associated with endosomal pathway, such as ESCRT machinery (e.g., Alix, TSG101), proteins involved in EV formation and release (e.g., RAB27A, ARF6), transcription factors (Notch and Wnt), surface receptors, growth factors, and cytokines [17]. In addition to EV-specific proteins, other ubiquitous cytoplasmic proteins such as cytoskeletal proteins

(e.g., actin, tubulin, actin binding proteins) can also exist in the cargo [18]. EVs also contain many common lipid composition, as well as differential expression in comparison to the cells of origin. EVs may actually contain increased amount of cholesterol, unsaturated lipids, sphingomyelin, ganglioside GM3, phosphatidylserine, and ceramide compared to parental cell itself [19, 20]. Moreover, EVs contain various genetic materials including mRNAs, which can be translated in recipient cells, microRNA (miRNA), and noncoding RNAs (ncRNA), which can regulate gene/mRNA expression, as well as genomic and mitochondrial DNAs [21–26]. Currently, there are web-based databases providing compendium on contents of EVs and are publicly accessible: EVpedia (http://evpedia.info), ExoCarta (exocarta.org), and Vesiclepedia (http://www.microvesicles.org). These databases include not only information on the nucleic acid, lipid, and protein content of EVs but also the isolation procedure used by different researchers.

Several mechanisms used by EVs for target cell internalization have been proposed. The EVs can be taken up by membrane fusion directly into recipient cells by clathrin-mediated endocytosis, caveolin-dependent internalization, and phagocytosis [27, 28]. Moreover, ligand-receptor interaction between proteins on EVs membrane and receptors on the recipient cell membrane may lead to stimulation of signaling cascades in the target cells [27, 29]. Upon uptake and transfer, biological cargos within EVs will be delivered and exploited in the recipient cells. For example, mRNAs can be translated into proteins or miRNAs can alter the gene expression profile of target cells [30]. Furthermore, the EVs contain multiple biomolecules that endow them with huge potential to transfer information into the recipient cells compared with known single molecule messengers, such as cytokines, growth factors, and other mediators [31, 32].

Paracrine action of stem-cell-derived EVs

In the last decades, tremendous interest has been focused on stem cells as therapeutic agent in regenerative medicine. Stem cells including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells have been investigated for their effects in various injured organs and tissues. Among them, mesenchymal stem cells (MSCs) are preferred cell types for use in regenerative therapies, because they can be readily collected and propagated, as well as differentiated into a variety of cell types. Currently, the focus on secreted EVs from stem cells has been most extensively directed to MSCs, and the most studied MSCs are those isolated from bone marrow (BMMSCs), adipose tissue (AD-MSCs), or umbilical cord blood (UC-MSCs) [33]. Moreover, compared to ESCs, they are free of ethical issues. Indeed, these multipotent MSCs when administered to treat animal models of myocardial infarction, stroke, acute kidney injury and diabetes demonstrated strong regenerative capabilities [34]. As an example, administration of BM-MSCs into experimental rodent models of acute kidney injury induced by ischemia reperfusion injury or toxic agents enhanced intrinsic kidney recovery, as well as improve kidney function [35]. It was initially thought that these beneficial effects were due to migration and engraftment of MSCs to the damaged kidney tissue and subsequent differentiation into tubule cells and vascular cells. However, it was later reported that the engrafted MSCs did not efficiently transdifferentiate, suggesting that therapeutic benefits of administered MSCs were not derived from differentiation into cell lineages replacing the injured tissues but mediated by paracrine mechanisms [36–40]. In these studies, treatment with MSCs enhanced proximal tubular cell proliferation, ameliorated apoptosis, and preserved microvascular integrity to improve renal tissue oxygenation. In addition, intraperitoneal injection of cell-free, MSC-conditioned medium was sufficient to stimulate the structural and functional regeneration of injured kidney tissue in cisplatin-induced AKI. This finding indicates that contribution of administered stem cells in restoring tissue is limited and that the paracrine secretion by MSC plays the major role in stimulating kidney tissue regeneration [41]. A key component of this paracrine mechanism is EVs, which mainly contribute to action of MSC in which genetic information, proteins, and lipids are transferred between MSCs and tissue injured cells. EVs derived from MSCs express proteins involved in MSC self-renewal and differentiation capability [42]. Moreover, these EVs also contain MSC-specific mRNAs, as well as microRNAs (miRNAs) that harbor-regenerative potential of MSCs [43]. Such therapeutic effects of EVs derived from diverse sources of stem/progenitor cells on regeneration of various tissues have been well documented in other recent publications [44–47]. Indeed, EVs derived from stem cells have demonstrated their ability to efficiently improve proliferation, angiogenesis, and immune modulation of injured tissues [48, 49]. Furthermore, within the EVs multiple different proteins and multiple types of coding and noncoding nucleic acids are packed and they may exhibit more efficient and longer-lasting response compared with those induced by individual molecules [50–52]. Therefore, stem-cell-derived EVs represent a relevant therapeutic option to regenerate injured kidney and in the following part, we summarized the roles that EVs play in various models of kidney injury (Table 13.1).

TABLE 13.1 Efficacy of stem-cell-derived extracellular vesicles in various kidney injury models.

Studies	Animal	Disease model	Stem cell source	Experiment method	Main results
Lindoso et al. [53]		In vitro model of IRI	BMMSC-EVs	In vitro incubation of renal PTECs in the presence or absence of EVs.	Enhanced incorporation of EVs into injured PTECs EV-mediated miRNA transfer and transcriptional modulation of miRNAs in injured PTECs Downregulation of miRNAs associated with apoptosis, cytoskeleton reorganization, and hypoxia
Gatti et al. [54]	Rat	Unilateral IRI	BMMSC-EVs	EVs were administrated via IV immediately after right nephrectomy and clamp of left renal pedicle for 45 min Rats were sacrificed at day 2 and 6 months after the operation	EVs inhibited TEC injury and apoptosis EVs significantly improved TEC proliferation and renal function EVs protected development of later chronic kidney disease
Collino et al. [55]	Mouse	Glycerol-induced AKI	BMMSC-EVs	EVs derived from Drosha-knockdown BMMSC (EV-Dsh) or BMMSC-EVs were administered via the tail vein 3 days after glycerol administration in SCID mice	EV-Dsh showed global downregulation of miRNAs Administration of miRNA depleted MSCs or EVs resulted in significant reduction in their intrinsic regenerative potential in AKI, suggesting a critical role of miRNAs
Ju et al. [56]	Rat	Unilateral IRI	UC-MSCs-EVs	EVs were IV injected into AKI animals via the vena caudalis immediately after IRI	EVs upregulated HGF expression in damaged rat tubular cells EVs mediated human HGF mRNA transfer into damaged rat tubular cells EVs decreased TEC apoptosis, collagen deposition and promoted cell proliferation to accelerate renal recovery
Cantaluppi et al. [57]	Rat	Unilateral IRI	EPC-EVs	EVs were injected in the tail vein immediately after IRI	EVs improved tubular cell proliferation EVs decreased tubular cell apoptosis, and leukocyte infiltration miRNA cargo of EVs contribute to reprogramming of hypoxic resident renal cells to a regenerative program
Shen et al. [58]	Mouse	Unilateral IRI	BMMSC-EVs	EVs were injected into a renal capsule in mice after IRI	EVs highly enriched in CCR2 decreased free CCL2 concentrations Administration of CCR2 enriched EVs modulate inflammatory processes to exert kidney repair
Zou et al. [59]	Rat	Unilateral IRI	WJ-MSC-EVs	EVs were injected intravenously in rats immediately after ischemia of the left kidney for 60 min	EVs reduced apoptosis and enhanced TEC proliferation followed by reduction in inflammation EVs improved renal function and abrogation of renal fibrosis
Gu et al. [60]	Rat	Unilateral IRI	WJ-MSC-EVs	EVs were injected intravenously in rats immediately after monolateral nephrectomy and renal pedicle occlusion for 45 min and sacrificed at 24 h	EVs inhibited mitochondrial fission and reduced cell apoptosis via miR-30
Choi et al. [61]	Mouse	Bilateral IRI	KMSC-EVs	KMSC-derived EVs were administered via tail vein injection, after bilateral renal ischemia	EVs, with genes for VEGF, bFGF, and IGF-1, incorporated into HUVEC and decreased apoptosis and increased cell proliferation, as well as tube formation EVs selectively engrafted into peritubular endothelial cells, increased endothelial cell proliferation and ameliorated peritubular capillary rarefaction EVs improved of kidney function
Tomasoni et al. [62]		In vitro model of cisplatin-induced nephrotoxicity	Human BMMSC-EVs	Cisplatin-pretreated PTECs were incubated with a test medium or human BMMSC-EVs or IGF-1 or both for 4 days	EVs increased PTEC proliferation EVs transferred IGF-1R mRNA into cisplatin-damaged PTEC Coincubation of damaged PTEC with EVs and soluble IGF-1 further enhanced cell proliferation

TABLE 13.1 Efficacy of stem-cell-derived extracellular vesicles in various kidney injury models—cont'd

Studies	Animal	Disease model	Stem cell source	Experiment method	Main results
Zhou et al. [63]	Rat	Cisplatin-induced AKI	UC-MSCs-EVs	Human UC-MSCs-EVs were injected into the kidneys via the renal capsule at 24 h after treatment with cisplatin All animals were killed at day 5 after administration of cisplatin	EVs inhibited tubular cell apoptosis and improved tubular cell proliferation EVs depressed renal oxidative stress EVs suppressed cisplatin-induced apoptosis and promoted proliferation in NRK-52E cells by activation of the ERK1/2 pathway in vitro
Wang et al. [64]	Rat	Cisplatin-induced AKI	UC-MSCs-EVs	Human UC-MSCs-EVs were injected into the kidneys via the renal capsule prior to treatment with cisplatin Rapamycin were injected under the kidney capsule before human UC-MSCs-EVs All animals were sacrificed at 3 days after cisplatin injection	EVs pretreatment inhibited apoptosis and secretion of inflammatory cytokines in renal TEC EVs activated autophagy that partly mediated renoprotective effects
Bruno et al. [65]	Mouse	Glycerol-induced AKI	BMMSC-EVs	SCID mice were injected intramuscular hypertonic glycerol followed by an intravenous injection into the tail vein of BMMSC-EVs on day 3 after glycerol administration	EVs induced proliferation of tubular cells via RNA-dependent biologic effects
Reis et al. [66]	Rat	Gentamycin-induced AKI	BMMSC-EVs	Daily injections of gentamycin up to 20 days with administration of BMSCs or BMMSC-EVs from the 10th day of gentamycin injections	EVs inhibited the impairment of renal function, necrosis, apoptosis, and increased cell proliferation EVs decreased pro-inflammatory cytokines while increased the antiinflammatory cytokines compared to gentamycin
He et al. [67]	Mouse	UUO	BMMSC-EVs	BMMSC-EVs were injected through caudal veins at day 2 after UUO injury The mice were sacrificed at day 7 and 14 after the first injection	EVs decreased tubular injury and epithelial to mesenchymal transition EVs significantly reduced degree of glomerulosclerosis and collagen content
Choi et al. [68]	Mouse	UUO	KMSC-EVs	KMSC-EVs were administered via the tail vein after UUO injury and mice were sacrificed at day 7 after surgery	EVs significantly inhibited endothelial-to-mesenchymal transition of peritubular capillary endothelial cells and improved peritubular capillaries rarefaction EVs inhibited inflammatory cell infiltration, enhanced tubular cell proliferation, and decreased tubulointerstitial fibrosis
He et al. [69]	Mouse	5/6 subtotal nephrectomy	BMMSC-EVs	BMMSC-EVs were injected through caudal veins at day 2, 3, 5 after 5/6 subtotal nephrectomy	EVs decreased renal fibrosis, inflammation, and tubular atrophy EVs prevented renal fibrosis and improved renal function
Grange et al. [70]	Mouse	Diabetic nephropathy	BMMSC-EVs and HLSC-EVs	EVs were administered weekly to streptozotocin induced diabetic mice for 4 weeks	EVs significantly ameliorated glomerular alterations and reduced tubular damage and improved renal fibrosis. EVs downregulated pro-fibrotic genes and EV cargo miRNA modulated fibrosis-related genes
Jiang et al. [71]	Rat	Diabetic nephropathy	USCs-EVs	EVs were administered weekly via tail intravenous injection until 12 weeks in streptozotocin induced diabetic rats	EVs prevent podocyte and tubular epithelial cell apoptosis, and increase glomerular endothelial cell proliferation EVs reduced the urine volume and urinary microalbumin excretion

EVs, extracellular vesicles; *MSCs*, mesenchymal stem cells; *IRI*, ischemia-reperfusion injury; *PTECs*, renal proximal tubular epithelial cells; *BMMSCs*, bone-marrow-derived mesenchymal stem cells; *AKI*, acute kidney injury; *UC-MSCs*, umbilical cord blood-derived mesenchymal stem cells; *EPC*, endothelial progenitor cells; *WJ-MSCs*, Whartońs Jelly derived mesenchymal stem cells; *KMSCs*, kidney-derived mesenchymal stem cells; *UUO*, unilateral ureteral obstruction; *HLSC*, human liver stem-like cells; *USCs*, urine-derived stem cells.

EVs in the treatment of experimental AKI

Ischemia-reperfusion injury

Acute kidney injury (AKI) is a syndrome characterized by a rapid decline in kidney function in parallel with loss of tubular cells, as well as a wide array of inflammatory responses [72]. Renal ischemia-reperfusion injury is one of the most common cause of AKI and is associated with significant morbidity and mortality [73]. The ischemia-reperfusion injury of the kidney is caused by sudden impairment of the blood flow to the kidney, with a subsequent reperfusion and re-oxygenation. The generation of reactive oxygen species at reperfusion can induce a robust inflammatory and oxidative stress response that leads to cell death and rapid deterioration of kidney function [74]. As such, multiple pathways are involved in activation and progression of AKI. Therefore, renal recovery processes necessary to repair cell damage and function should be multifaceted.

The endogenous repair of damaged kidney cells from ischemia-reperfusion injury is one therapeutic area of EVs application. Indeed, EVs derived from BMMSCs (BMMSC-EVs) protected renal tubular epithelial cells (TECs) in an in vitro model of IRI induced by ATP depletion. Treatment with the BMMSC-EVs effectively protected the renal TECs by decreasing TUNEL-positive apoptotic TECs, and this protective effect was associated with EV-mediated miRNA transfer, as well as transcriptional modulation of miRNAs expressed by injured TECs. In particular, coding-mRNAs associated with apoptosis, cytoskeleton reorganization, and hypoxia were downregulated [53]. Similarly, treating rodent model of AKI with BMMSC-EVs supported the in vitro data, leading to a decrease of TUNEL-positive cells and an increase of BrdU-positive cells in the kidney. A single administration of BMMSC-EVs, immediately after ischemia-reperfusion injury, protected rats from AKI by inhibiting apoptosis and stimulating TEC proliferation resulting in significant improvement of renal function. The underlying mechanism of actions of EVs, at least in part, are due to uptake of EVs by damaged TECs, as shown in vivo a few hours after administration using labeled EVs and the transfer of biological components such as mRNA and microRNAs (miRNAs) into recipient TECs. Pretreatment of BMMSC-EVs with RNase to inactivate their RNA cargo reduced these protective effects implying that mRNA- or miRNAs-mediated EVs-induced renal repair [54]. Microarray analysis of the EVs detected 239 species of mRNA molecules, with specific enrichment of mRNAs involved in cell proliferation, transcription, and immune regulation [65]. In addition to mRNAs, the miRNAs carried by EVs derived from stem cells also have been found to demonstrate a relevant protective role in experimental kidney injury. The miRNAs are a class of small noncoding RNAs that are involved in posttranscriptional modulation of gene expression and regulate important cellular processes [75]. In ischemia-reperfusion AKI models, various miRNAs are reported to regulate major pathways of apoptosis, inflammation, fibrosis, and cell cycle such as phosphatase and tensin homolog (PTEN), heme oxygenase (HO-1), forkhead box O3 (Foxo3), hypoxia-inducible factor (HIF)-1, and phosphoinositide 3 kinase (PI3K), indicating the pivotal function of miRNAs in AKI [76]. The therapeutic role of miRNA was further clarified using Drosha-knock down BMMSCs. The Drosha, a double-stranded RNA-specific ribonuclease, is an important enzyme in miRNA synthesis. RNA molecules generated by the transcription are cleaved by Drosha into precursor miRNAs (pre-miRNAs) in the nucleus. EVs derived from these Drosha-knock down BMMSCs showed global downregulation of microRNAs. Whereas administration of wild-type BMMSCs and their EVs induced morphologic and functional recovery in AKI, the miRNA-depleted EVs isolated from Drosha-knock down BMMSCs failed to improve AKI [55]. Such findings suggest a critical role of miRNAs in recovery after AKI. The protective functions of individual miRNAs in AKI, such as miR-10a, miR-21, miR-26a, miR-126, miR-146a, miR-199a, and miR-296 have also been reported [77].

Several preclinical studies have demonstrated that EVs derived from various sources of stem cells promote tissue repair and ameliorate inflammation in ischemia-reperfusion AKI. EVs derived from human UC-MSCs (UC-MSC-EVs) stimulated TEC proliferation by the horizontal transfer of human HGF and by the induction of hepatocyte growth factor (HGF) production. Administration of UC-MSC-EVs into rats with ischemia-reperfusion AKI resulted in demonstration of human HGF mRNA in damaged rat tubular cells, indicating mRNA transfer. The transferred human HGF mRNA was subsequently translated into the corresponding protein. High HGF levels activated the ERK1/2 signaling pathway, while inhibited cell apoptosis, decreased collagen deposition, and promoted cell proliferation that accelerated renal recovery [56]. In addition, the beneficial effect of HGF mRNA transfer by conditioned media from human Whartoń's-jelly-derived MSC (WJ-MSC) was observed in an AKI-CKD transition model by the same group. Administration of conditioned media isolated from WJ-MSC lead to recovery of disturbed balance between HGF and TGFbeta1 during fibrosis process and alleviated renal fibrosis [78].

EVs derived from endothelial progenitor cells (EPC-EVs) enhanced TEC proliferation and reduced apoptosis and leukocyte infiltration in renal ischemia-reperfusion injury. Moreover, the EPC-EVs contained angiogenic miRNAs, miR-126 and miR-296, which were associated with triggering of angiogenesis of peritubular endothelial cells. Thus,

EPC-EVs protected kidney by delivering their angiogenic miRNA cargo and reprogramming of injured hypoxic renal cells to a regenerative program [57].

Inflammation plays an important role in the pathogenesis of AKI. Activation and subsequent tissue infiltration of various inflammatory cells, especially macrophages, is pivotal in development of AKI [79]. Therefore, reducing activation and infiltration of macrophages may be a good potential target for AKI treatment. The CCL2, one of the most important chemokines contributing to the proinflammatory macrophage recruitment, and its major receptor C–C motif chemokine receptor-2 (CCR2) are the key factors in regulation of the tubular injury after ischemia-reperfusion-induced AKI [80]. In addition to transferring RNA or miRNA to target cells, BMMSC-EVs were found to function as carriers of CCR2. The administration of CCR2-enriched EVs strongly bound the extracellular CCL2 and decreased free CCL2 concentrations that subsequently inhibited the recruitment or activation of macrophages in kidney. In addition, the EVs derived from CCR2 knockdown BMMSCs failed to exert protective effects in ischemia-reperfusion-induced AKI. These data indicate that treatment with CCR2 is an important molecule on BMMSC-EVs, which modulates inflammation processes and acts as a decoy for CCL2 suppression to exert kidney repair [58]. Another study, using EVs derived from human WJ-MSCs, demonstrated mitigation of renal TEC apoptosis and enhanced TEC proliferation followed by reduction in inflammation in AKI rats. WJ-MSC-EVs also suppressed the expression of CX3CL1 and decreased the number of CD68 + macrophages in the kidney. Two weeks later, improvement of renal function and abrogation of renal fibrosis were observed, suggesting antiinflammatory property of WJ-MSC-EVs via downregulation of CXCL1 expression [59].

Mitochondrial dysfunction is a pathological consequence of ischemia-reperfusion injury. Therefore, promoting mitochondrial biogenesis can be a unique strategy to restore mitochondrial and renal function [81]. Under ischemia-reperfusion stress, mitochondrial dynamics is shifted to excessive fission state, leading to mitochondrial fragmentation and apoptosis [82]. Recent study identified a miR-30-related apoptotic pathway in AKI that involves mitochondria and dynamin-related protein 1 (DRP1), a key regulator of mitochondrial fission. Administration of WJ-MSC-EVs enhanced miR-30b/c/d of renal tubular cells and mitigated the activation of DRP1, as well as mitochondrial fragmentation and apoptosis. These results demonstrate that WJ-MSC-EVs may ameliorate ischemia-reperfusion AKI by inhibition of mitochondrial fission via miR-30 [60].

Modulation of renal angiogenesis by delivered MSC-EVs is another mechanism of renoprotection. EVs derived from kidney MSC (KMSC-EVs) were found to contain genes for vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1). Intravenous administration of KMSC-EVs in mice with ischemia-reperfusion AKI was followed by homing and engraftment of EVs into peritubular endothelial cells and amelioration of microvascular rarefaction and improvement of kidney function. The renoprotective effects of KMSC-EVs were abolished when these EVs were pretreated with RNase, suggesting that transfer of proangiogenic mRNAs-mediated protective effects of KMSC-EVs [61]. Similarly, administration of UC-MSC-EVs in rats with ischemia-reperfusion AKI showed increase in renal capillary density, as well as reduction of renal fibrosis by direct transfer and expression of proangiogenic VEGF and mRNA [83]. Interestingly, repeated injections of EVs derived from BMMSCs prevented development of renal fibrosis after ischemia-reperfusion injury [54].

Collectively, these results indicate that EVs derived from MSC of various tissues are effective in stimulating cell proliferation, inhibiting apoptosis, inflammation, and oxidative stress to improve ischemia-reperfusion AKI.

Drug-induced AKI

Drug-induced nephrotoxicity is one of the major causes of AKI, especially in hospitalized and elderly patients, who are exposed to numerous medications [84]. Nonsteroidal antiinflammatory drugs, antimicrobial agents, chemotherapeutic agents such as cisplatin, and radiocontrast agents used are associated with development of AKI. Pathophysiology of drug-induced AKI is complex and involve derangement of intraglomerular hemodynamics, tubular cell toxicity, inflammation, and crystal nephropathy that lead to apoptosis, necrosis, and other modes of cell death [85]. Recent studies have shown that EVs derived from MSC have beneficial effects in the treatment of drug-induced nephropathy.

Cisplatin is a widely used chemotherapeutic drug in the treatment of many solid organ cancers. The main dose-limiting side effect of cisplatin is nephrotoxicity. The cisplatin-induced nephrotoxicity involves multiple pathways including oxidative stress and activation of apoptotic cascades that lead to impairment of renal function. In addition, cisplatin induces release of damage-associated molecular pattern molecules (DAMPs) from TECs that promote production of a variety of chemokines and cytokines and the induction of an inflammatory response in the injured kidney [86]. BMMSC-EVs conferred protection against cisplatin induced renal injury. Coincubation of cisplatin-damaged TECs with BMMSC-EVs increased cell proliferation, partly by transfer of mRNA for IGF-1

receptor to cisplatin-damaged TECs and potentiation of tubular cell sensitivity to actions of IGF-1 [62]. These observations were supported by in vivo studies in murine models of drug-induced nephropathy, in which the administration of human UC-MSC-EVs improved cisplatin-mediated renal damage. The UC-MSC-EVs treatment attenuated renal injury partly by reducing formation of prooxidants and suppressing activation of proapoptotic pathways leading to decreased oxidative stress and apoptosis and enhanced cell proliferation by activation of the extracellular-signal-regulated kinase (ERK)1/2 pathway [63]. Similarly, the UC-MSC-EVs treatment inhibited mitochondrial apoptosis and secretion of inflammatory cytokines in TECs and increased the expression of the autophagic marker protein LC3B and the autophagy-related genes ATG5 and ATG7 in NRK-52E cells. The study also suggest that the antiapoptotic and immunomodulatory effects of UC-MSC-EVs in drug-induced AKI are partly mediated by their ability to transfer genes that activate autophagy [64].

Moreover, BMMSC-EVs displayed a therapeutic role in accelerating recovery of glycerol-induced AKI in SCID mice, as well as improving survival in a cisplatin-induced lethal AKI, mimicking the effects of their parental cells. In particular, treatment with the BMMSC-EVs effectively ameliorated tubular injury and improved kidney function. Modulation of apoptosis was implicated in BMMSC-EVs induced renoprotective effect, which was abolished after degradation of mRNA content of BMMSC-EVs, suggesting that transfer of antiapoptotic gene cargos by BMMSC-EVs are the final effectors of their renoprotective actions [65, 87].

Modulation of renal inflammation is another important mechanism by which BMMSC-EVs protect the kidney from toxic drug injury. Gentamycin-induced AKI is characterized by increased oxidative stress and activation of inflammatory pathway. Administration of BMMSC-EVs improved kidney function by preventing increase in levels of proinflammatory cytokines, including IL-6 and TNF-alpha, whereas levels of the antiinflammatory cytokine IL-10 were increased [66]. Likewise, in mice with glycerol-induced AKI, BMMSC-EVs delivery was associated with down-regulation of pro-inflammatory genes [55].

The renoprotective effects of BMMSC-EVs were blunted when mice were treated with RNA-depleted BMMSC-EVs, indicative of an important role for mRNA and/or microRNA cargos in mediating recovery of kidney function after AKI. Taken together, these results suggest that EVs are capable of modulating several pathways involved in the pathogenesis of drug-induced nephropathy and may be exploited as novel therapeutic agents.

EVs in the treatment of experimental CKD

Chronic kidney disease (CKD) is characterized by progressive tubulointerstitial fibrosis accompanied by reduced regenerative potential that represents a potential treatment target. Several studies suggest beneficial regenerative effects of stem-cell-based therapies in experimental models of CKD [88]. Apart from AKI, EVs derived from stem cells have also demonstrated to be therapeutically effective in experimental models of CKD similar to their parental cells.

Unilateral ureter obstruction

The unilateral ureter obstruction (UUO) promotes renal parenchymal inflammation, apoptosis, and fibrosis and is one of most widely used experimental models of CKD [88, 89]. EVs derived from BMMSCs contained selective patterns of miRNAs that may be transferred to target renal cells to modulate fibrosis and epithelial to mesenchymal transition (EMT). In vitro experiments where TECs are treated with the profibrotic transforming growth factor (TGF)-β1 showed that coincubation with EVs derived from BMMSCs reversed EMT and TGF-β1-induced morphological changes. Intravenous administration of EVs derived from BMMSCs ameliorated tubular injury and interstitial fibrosis and improved renal function 2 weeks after UUO [67]. Another study using EVs derived from kidney MSCs also demonstrated similar results. In vitro experiments where human umbilical vein endothelial cells are treated with TGF-β1 showed that EVs derived from kidney MSCs ameliorated endothelial to mesenchymal transformation (EndoMT) and improved cell proliferation 7 days after UUO [68]. These results underscore important antifibrotic and renoprotective properties of EVs derived from MSCs in experimental UUO.

Subtotal nephrectomy

Subtotal nephrectomy, so-called 5/6 nephrectomy, is another commonly used experimental model of CKD. It is characterized by widespread glomerulosclerosis and tubulointerstitial fibrosis and progressive loss of renal mass that results in severe deterioration of renal function [90]. The 5/6 nephrectomy mice treated with multiple intravenous injection of EVs derived from BMMSCs (day 2, 3, 5 after the surgery) demonstrated relatively well-preserved kidney

architecture and significant reduction of the histologic features of renal injury. EVs-treated mice kidney showed less interstitial lymphocyte infiltration, tubular swelling and necrosis, glomerulosclerosis, and interstitial fibrosis. Moreover, the levels of serum creatinine, proteinuria, and serum uric acid levels decreased significantly after administration of EVs. Such data indicate that treatment with EVs at the onset of the injury might limit the aggravation of initial injury and mitigate the development of serious renal impairment [69].

Diabetic nephropathy

Diabetic nephropathy is a microvascular complication of diabetes and histologically characterized by glomerular and tubulointerstitial fibrosis. Fibrotic process is the major cause of the pathogenesis of diabetic nephropathy. The epithelial-to-mesenchymal transition (EMT), activation of myofibroblasts, and excessive deposition of extracellular matrix molecules are crucial processes involved in pathogenesis of diabetic nephropathy. Therefore, regulation of fibrosis is a potential target of treatment. EVs derived from MSCs have been reported to modulate proliferation, angiogenesis and immune escape, which are all involved in tissue regeneration [91–93].

Direct administration of EVs derived from BMMSCs into the renal subcapsular space of STZ-induced diabetic rats induced a rapid improvement of kidney tissue histology. EVs reduced TECs apoptosis and suppressed abnormal bone-marrow-derived cell (approximate 50% are F4/80 positive macrophages) infiltration in the kidney. The same investigators showed that treatment with conditioned medium derived from MSCs, that contains abundant quantities of EVs, suppressed tubulointerstitial fibrosis by improving excessive expression of proinflammatory cytokines [94]. Recently, EVs derived from human BMMSCs or human liver stem-like cells (HLSCs) have shown to be beneficial in preventing and reversing progression of diabetic nephropathy induced by streptozotocin in mice. In contrast to previous study [94], therapeutic effects of EVs derived from stem cells were studied after the development of diabetic nephropathy and administration of these EVs significantly improved renal functional parameters. Interestingly, the antifibrotic effects of EVs derived from BMMSC and HLSC correlated with the downregulation of several profibrotic genes in renal tissues [70]. Moreover, EVs isolated from urine derived from urine stem cells also prevented kidney injury by inhibiting podocyte apoptosis and promoting vascular regeneration when administered before the establishment of diabetic nephropathy in a streptozotocin injected rat model of diabetes [71].

In summary, recent studies have shown that administration of EVs isolated from various MSCs have potential to promote kidney repair in experimental models of AKI and CKD. These MSC-derived EVs contain genetic and protein cargo that upon transferring to recipient cells can modulate several repair mechanisms. In particular, administration of MSC-derived EVs significantly ameliorated injuries to renal tubular cells, modulated inflammatory responses, prevented tubulointerstitial fibrosis, and improved microvascular rarefaction, as well as kidney function. However, further basic and translational studies are needed to better establish the functional significance of stem-cell-derived EVs in kidney diseases.

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CHAPTER

14

Stem cell therapies in diabetes

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Introduction

Affecting more than 10% of Americans, diabetes is currently the most prevalent metabolic disease, with over 1 million new cases diagnosed each year in the United States [1]. It is a chronic and complicated disorder characterized by a lack of natural glucose regulation in the blood, by attenuation of either insulin response or insulin production. When insulin is unable to effectively signal for glucose uptake to various tissues of the body, glucose builds to dangerous levels in the bloodstream, also known as hyperglycemia. Although transitory hyperglycemia can be harmless, strongly acute levels of glucose can cause immediate medical issues similar to that of chronic hyperglycemia [2]. Vascular tissue is the most affected by glucose toxicity, leading to the classical symptoms of neuropathy on the microvascular scale, or cardiovascular complications on the macrovascular scale [3]. Notably, complications in the kidney from diabetes can be severe, ranging initially from dehydration and osmotic diuresis to nephropathy and eventually kidney failure. About 40% of patients with diabetes develop some type of kidney disorder, and the leading cause of chronic kidney disease is diabetes [4].

Diabetes is categorized into two main types, depending on the origin of metabolic malfunction. Type 1 diabetes (T1D), prevalent in 5%–10% of patients with diabetes, is brought on by an autoimmune attack against the insulin-producing cells of the pancreas: the β cells. As β cells die or become damaged, their overall capacity for insulin production is greatly diminished, leading to a loss of glucose control in the blood stream [5]. Without the ability to produce enough, or any, of their own insulin, patients with type 1 diabetes rely on insulin injections to stabilize blood sugar levels. If left unchecked, the chronic low levels of insulin can lead to catabolic changes in the body to signal for lipolysis and ketone production. The buildup of ketones in the blood eventually leads to diabetic ketoacidosis [6].

Type 2 diabetes (T2D) comprises the other 90%–95% of patients with diabetes, and is caused by building resistance of the body to the insulin signal. There are a number of complicating factors in discovering the initial catalyst of the disease, including genetic background, diet, obesity, intrauterine development, and pharmacological side effects [6, 7]. However, all patients present with insulin resistance as well as, due to the ever-increasing demand, insulin deficiency from the stress of overproduction on the β cells. As hyperglycemia worsens, the β cells become further damaged by the glucose toxicity, exacerbating the cycle of metabolic aberration [7]. Since insulin insensitivity is the primary factor, insulin injections are generally used as a late stage treatment, secondary to lifestyle and diet changes [8].

We have made great strides in diabetes care since the first administration of insulin in 1922. Previously, the only known treatment was an incredibly strict diet and exercise routine which left most patients deceased just a few years after onset. Currently, there are several options for diabetes treatment which provide the patient vastly decreased morbidity, with most estimates showing diabetes to reduce life span by about a decade or so. For nearly 100 years, insulin remains vital for the treatment of type 1 and some type 2 diabetic patients, allowing for instant control of blood glucose levels on an as-needed basis. Alternatively, the use of an insulin pump provides a steady dose of insulin as well as an easy method for administering larger doses at meal times to give greater blood sugar control while diminishing the burden of injections for the patient. The integration of a continuous glucose monitor greatly increases the accuracy and efficacy of the insulin pump system by customizing insulin dose through constant checks of blood glucose levels. Oral medications such as metformin also assist patients with type 2 diabetes by decreasing the glucose

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production of the liver and increasing insulin sensitivity. For type 1 patients with difficulty to manage symptoms, pancreas and islet transplantation are sometimes experimentally employed to help replenish the natural insulin production of the body. Finally, exercise and a planned diet contribute substantial support for all patients with diabetes, as exercise increases insulin sensitivity while a well-structured diet can help control blood sugar spikes after eating.

Due to soaring costs of insulin and the difficulty of major lifestyle changes, other more reliable approaches for the treatment of diabetes are preferable. Since the major point of failure in the diabetic metabolism is the β cell, transplantation has been an attractive route to restore insulin function. However, aside from issues with availability of the tissue, transplantation of β cells comes with a host of difficulties from the delicate nature of the cells themselves [9]. Since β cells work alongside a consortium of other pancreatic cells in small clusters known as islets, the whole islet must be transplanted to ensure proper proportions of β cells to the other cells of the pancreas. Considering the pancreas is a highly vascular organ with intricately structured microanatomy, this is not an easy task. Full pancreas transplants are most often performed in conjunction with necessary kidney transplants since the procedures carry similar risks.

Islet cell transplantation

Currently, β-cell replacement therapy is one of the major focuses to achieve glucose homeostasis. Several trials, especially following the Edmonton protocol, have shown success in graft function, absence of hypoglycemic events, and normalization of blood glucose levels. Although studies have shown improved glycemic control following islet transplants in patients with type 1 diabetes, the positive effects diminish so rapidly that after just 5 years more than 90% of patients still required an insulin treatment regimen [10]. In addition, patients with donor islets require immunosuppressive drugs to ward against graft rejection, which only aggravates the already present issues of increased infection risk, increased blood glucose, and kidney damage. Follow-up research showed a reported better health status overall, however, even for those who continued to take insulin after surgery, so improvements on islet survival posttransplantation are a promising avenue for continuing treatment [11]. Besides, the availability of pancreatic human islets and the isolation protocols to obtain high quality and yield is progressing but still needs improvements. Islet yield, purity, and cell viability are important factors that affect the β-cell replacement process. Currently, there are programs that coordinate the distribution of human islets for research or clinical transplantations.

Mesenchymal stem cells

As our understanding of cell biology improves, the prospect of regenerative therapy grows as an exciting tool for treating difficult to manage diseases. The use of one's own cells to repair damaged systems in the body has the exclusive benefit of avoiding a negative immunological response, in addition to providing the exact biological instrument necessary to mend the issue. In the scope of diabetes, mesenchymal stem cells (MSCs) have proven to be an auspicious tool in restoring insulin production for both type 1 and type 2 diabetic patients.

MSCs are multipotent stem cells which can be derived from a variety of areas in the adult body, such as bone marrow, skin, or bodily fluids, as well as in large amounts from the placenta and umbilical cord. Aside from their availability, they exhibit great benefits from their regenerative and immunomodulatory properties. First, they are able to differentiate into islet-like cells producing insulin in response to glucose, which could assist with the diminishing insulin production in patients with diabetes. They also secrete a great deal of cytokines which provide extensive benefits to the β cell. Signals for cell growth, immune suppression, angiogenesis, and antiapoptosis from transplanted MSCs directly combat some of the more debilitating symptoms of diabetes at the cellular level [12]. These have been shown to assist in promoting both self-regeneration of and transdifferentiation to the β cell in recent studies. Via immunoregulation, MSC factors also protect β cells by suppressing T-lymphocyte proliferation, thereby diminishing the immune attack in type 1 diabetes. Further, the expressed antiinflammatory signals have demonstrated a bolstered defense for β cells against oxidative stress and hypoxia (often caused by hyperglycemia) when cocultured with MSCs [13]. Some data have shown introductions of MSCs or simply their secreted factors have even supported an increase in insulin production of endogen β cells [14]. The use of conditioned media alone to treat β cells is particularly useful as MSCs are much smaller than islets, and so the logistics of cotransplantation of them both is hindered by MSC migration [15].

However, MSCs have displayed some disappointing results in recent in vivo and clinical trials (Table 14.1). After MSC transplantation in mice, it was found that very few, if any, MSCs were differentiating into β cells, and the regeneration of new β cells must be sourced from elsewhere [16, 17]. Injection of MSCs was unable to directly rescue glycemic control, though it assisted in other treatment methods like reducing islet numbers needed for transplants [18].

TABLE 14.1 Use of MSCs in clinical trial to treat diabetes.

Study	Duration	*n*	Country	Type	Delivery	Adverse reactions?	Improvements in experimental group?
Vanikar et al. [21]	2 years	11	India	T1D	Intraportal infusion	No severe reactions	Decreased exogenous insulin requirements, free of DKA events
Jiang et al. [22]	6 months	10	China	T2D	Monthly intravenous infusions for 3 months	No severe reactions	Improved average insulin dosage, C-peptide, and HbA1c
Hu et al. [23]	2 years	15	China	T1D	Once intravenous infusion	No severe reactions	HbA1c, insulin dosage, and C-peptide improvement, no difference in fasting blood glucose
Mesples et al. [24]	1 year	3	Argentina	T1D	Transplantation into liver	No severe reactions	Reduction in antiislet antibodies and HbA1c, and increased C-peptide
Liu et al. [25]	1 year	22	China	T2D	Once intravenous, then once intrapancreatic	Three patients with postoperative fever, one patient with subcutaneous hematoma, one patient with nausea, vomiting, and headache	Mild improvements in HbA1c, insulin dosage, and fasting C-peptide, systemic inflammation markers decreased at 6 months
Bhansali et al. [26]	15 months	11	India	T2D	Intrapancreatic infusion	No severe reactions	60% of patients showed reduction of mean insulin requirements and slightly improved HbA1c and C-peptide levels
Thakkar et al. [27]	2 years	20	India	T1D	Infused into portal circulation, thymus and abdominal subcutaneous tissue	No severe reactions	Sustained improvement in mean insulin requirement, serum C-peptide, mean HbA1c
Dave et al. [28]	3 years	10	India	T1D	Infused into portal circulation, thymus and into subcutaneous tissue	No severe reactions	Improved C-peptide, Hb1Ac, blood sugar status, and exogenous insulin requirement
Carlsson et al. [29]	1 year	9	Sweden	T1D	Once intravenous infusion	No severe reactions	AUC for C-peptide values (after meal tolerance test) in MSC group were preserved/increased
Skyler et al. [30]	12 weeks	45	United States	T2D	Once intravenous infusion	One patient with severe abdominal pain	One third of patients achieved HbA1c < 7%
Hu et al. [31]	3 years	31	China	T2D	Intravenous infusions monthly for 2 months	No severe reactions	Improvements in C-peptide and insulin dosage, mild benefits to HbA1c and fasting blood glucose
Cai et al. [32]	1 year	21	China	T1D	Intrapancreatic infusion	No severe reactions	Less hypoglycemic events, C-peptide AUC improved by 106%, serum insulin AUC increased 49%, HbA1c, FBG, insulin dose levels decreased
Bhansali et al. [33]	1 year	20	India	T2D	Intrapancreatic infusion	No severe reactions	Significant reduction in insulin requirement and improvement in insulin sensitivity index

Chart summarizing published data regarding the use of mesenchymal stem cells in the treatment of type 1 and type 2 diabetes. Ongoing clinical trials with yet unpublished data may be found online at ClinicalTrials.gov using search terms "diabetes" (under disease) and "mesenchymal cells" (under other terms).

Also, the origin of MSCs seems to play a vital role in their effectiveness, going so far as to contribute to the progression of diabetes when derived from patients with diabetes [19]. Despite these setbacks, MSC use has generally shown positive data in assisting patients, especially alongside other treatments. Although most studies struggle to show significance from small sample sizes or transient data, the results demonstrate a clear trend of improvement across patients with varied types of MSC infusions [20].

Human pluripotent stem cells

Human pluripotent stem cells (hPSCs) have an unlimited proliferative capacity that makes them excellent cell source for cell replacement therapies. These include human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSC). Although hESCs and hiPSCs are derived from different sources, both have the potential to differentiate into many different cell types in the body [34]. In 1998, hESC lines were generated for the first time by isolation of the inner cell mass of embryos that were produced by in vitro fertilization for clinical purposes and donated by individuals [35]. To date, 485 hESC lines are approved by National Institutes of Health (NIH) under the NIH guidelines for human stem cell research (http://www.nih.gov).

With the discovery of reprogramming, now somatic cells can be converted to a pluripotent state and provide an alternative for hESCs. First iPSCs were generated in 2006 by overexpression of four Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc) in mouse embryonic fibroblasts [36], and a year later the same approach was used to generate human iPSCs from human fibroblasts [37]. The use of integrating vectors such as retroviruses or lentiviruses for reprogramming brings safety concerns regarding the risk of malignant transformation. Therefore, other methods have been favored to deliver reprogramming factors to somatic cells such as Sendai virus, mRNA, and protein.

Generation of clinical-grade hPSCs

Following generation of hiPSC lines, cells are evaluated for several criteria to ensure the proper quality and safety suited for clinical and translational research [38,39] (Fig. 14.1). One of the criteria in the development of clinical-grade hiPSCs is to fully characterize cell lines, including assessment of pluripotent gene expression and spontaneous or directed differentiation potential to evaluate potency. Since reprogramming and in vitro culture conditions can induce genetic changes that could be harmful, hiPSCs are also tested to rule out any genetic abnormalities and oncogenic mutation. Other assays include microbiological sterility to prove that there is no contamination with pathogens, and short tandem repeat analysis to exclude culture cross-contamination. Another important criterion for the establishment of clinical-grade hPSC lines is to use defined culture system, free of xenogeneic agents such as fetal bovine serum and feeder cells [40].

Differentiation of hPSCs in vitro

Since hPSCs have the ability to differentiate any cell type in the body including kidney cells, cardiomyocytes, and pancreatic β cells, several groups focused their efforts to develop a differentiation protocol to generate disease-relevant cells from hPSCs. Elucidation of important signaling pathways regulating tissue development led researchers to mimic in vivo organogenesis in vitro. The directed differentiation approach involves guiding stem cells toward specific lineages by using small molecules and growth factors to activate/inhibit regulatory pathways and induce expression of key transcription factors.

Generation of kidney cells from hPSCs

Cells of the kidney can be generated by recapitulating key stages of renal embryonic development. The hPSCs are guided toward stages of late primitive streak, intermediate mesoderm, and metanephric mesoderm which gives rise to pretubular aggregates and then renal vesicles. Several groups have established differentiation protocols for

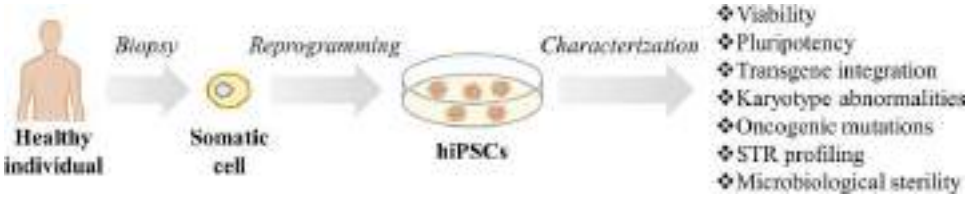

FIG. 14.1 Overview of generation of hiPSCs. Somatic cells such as fibroblasts or peripheral blood mononuclear cells are reprogrammed into hiPSCs and evaluated for several criteria to ensure their quality and safety are suited for clinical and translational research. These quality control steps include evaluation of viability and pluripotency of hiPSCs, establishing purity and identity by STR (short tandem repeat) profiling, and excluding pathogen contamination, transgene integration, karyotype abnormality, or oncogenic mutations. The hiPSCs that pass quality control are stored in cell banks for later use.

induction of renal progenitors [41]. Recently, hiPSC-derived renal progenitors have been shown to reduce kidney damage when implanted into diseased kidneys of an acute kidney injury mouse model [42,43]. These studies demonstrate the therapeutic efficacy of cell therapy in kidney diseases. Although the use of hiPSC-derived renal progenitors in regenerative medicine is very attractive, the development of such approaches is in its infancy. Currently, kidney organoids are mainly used for disease modeling and drug screening purposes [44].

Generation of pancreatic β cells from hPSCs

As for pancreatic β cells, stepwise differentiation protocols involve direction of stem cell fate into definitive endoderm, primitive gut tube, posterior foregut, pancreatic progenitors, pancreatic endocrine progenitors, and ultimately insulin-producing β-like cells. Several research groups published successful differentiation protocols to generate functional pancreatic β-like cells in vitro [45–47], and follow-up studies refined the protocols to improve the function and yield of β-like cells [48–50]. Although the β-like cells generated by using these protocols exhibit functional response, express key markers of β cells, and reverse hyperglycemia when transplanted to diabetic mice, they did not resemble fully mature human β cells from cadaveric islet donors. A key functional feature of β cells is their ability to respond repeatedly to a series of glucose challenges. Excitingly, recent studies have succeeded to generate β-like cells which possess dynamic insulin secretion properties similar to that of primary human islets [51,52]. Currently, hPSC-derived pancreatic progenitors have advanced to the clinical stage and they are being used in phase I/II clinical trial (NCT02239354). This trial aims to test if implantation of hESC-derived pancreatic progenitors is safe and effective for treatment of patients with type 1 diabetes, and the results are expected to be published soon.

Enrichment of differentiated cells derived from hPSCs

Cell therapy using differentiated cells from hPSCs has the potential to treat patients with diabetes (Fig. 14.2). However, the differentiation cultures used may contain undesired cell types that have high proliferation potential and could develop into tumors after transplantation. Safety of the final cell product is one of the major concerns to be addressed in hPSC-based transplantation therapy. Possible solutions include the elimination of unwanted cells from differentiation cultures, strict selection of the differentiated end-stage cells, and expansion of the therapeutic cells.

(a) Elimination approach: One of the approaches to eliminate tumor-initiating cells is to harness suicide genes such as the herpes simplex virus thymidine kinase gene (HSV-TK). This approach involves genetically engineering hPSCs to insert an inducible suicide gene into the genome. By treating with ganciclovir, the cells carrying HSV-TK gene can be killed even after cell transplantation [53]. Although this safeguard system is an effective way to kill tumor-initiating cells, CRISPR-mediated genome editing could introduce additional challenges such as off-target editing, and could raise safety concerns regarding clinical approval. Another approach to eliminate unwanted cells is to use a specific media formulation to kill undifferentiated cells without harming other cells [54].

(b) Selection approach: Specific cell surface markers are widely used to purify a target cell population. Hoshina et al. screened monoclonal antibodies against cell surface markers that are specific to OSR1 + SIX2 + renal progenitors and identified a combination of four markers CD9 −, CD140a +, CD140b +, and CD271 + that could enrich renal progenitors [55]. As for the pancreatic β cells, two research groups independently identified glycoprotein 2 (GP2) as a marker for pancreatic progenitors [56,57]. Selection and further differentiation of GP2 + pancreatic progenitors into β-like cells increased the yields. While this method allows selection

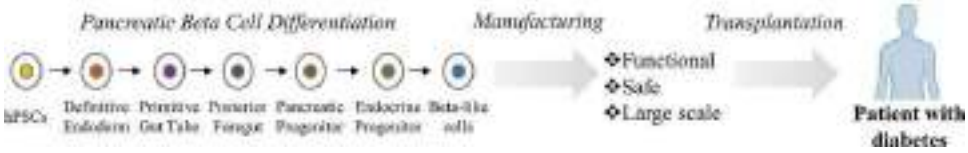

FIG. 14.2 Using stepwise protocols, hPSCs can be differentiated toward pancreatic β-like cells. Bringing cell therapies to the clinic requires generation of differentiated end-stage cells that are safe, functional, and at a scale sufficient to replace damaged cells. The end-stage differentiated cells includes non-β nonendocrine cells in addition to β-like cells. In order to obtain highly pure populations of endocrine cells, purification of β-like cells and elimination of non-β nonendocrine cells with high proliferation potential are possible alternatives. The products of β-like cells need to be manufactured at the clinical scale to achieve insulin independence after transplantation into patients with diabetes.

of pancreatic progenitors, further differentiation of sorted pancreatic progenitors into β-like cells results in a heterogeneous cell population. In searching for specific cell surface markers for β-like cells, CD49a, also known as ITGA1, was identified as a surface marker of the β-like cells which allows magnetic sorting to a purity of 80% [58]. More recently, CD9 was described as a negative selection marker to eliminate CD9+ non-β cells [59]. Additional approaches for enrichment is to utilize the exceptionally high zinc concentration of insulin expressing β cells to purify β-like cells in differentiating cultures [60]. The use of zinc-binding fluorescence probes allows enrichment of β-like cells and holds an advantage over the cell surface antibody approach since it is a very rapid, inexpensive, sensitive, and reproducible method [61].

(c) Expansion approach: The cell enrichment strategy aims to maximize β-like cell production followed by selection of pure cells for transplantation. One method of purification using cell surface antibodies requires fluorescence-activated cell sorting which limits large-scale production of β-like cells. Magnetic cell sorting could similarly be used to sort a larger number of cells. Selectively expanding β-like cells could efficiently increase yields, as long as cell quality is not compromised. This can be accomplished by development of a targeted small molecule delivery strategy. Proliferation inducing small molecules can be delivered specifically to β cells through a prodrug strategy, where an inactive compound is converted to the active agent only in the target cell by an enzyme [62].

Scalable manufacturing of differentiated cells derived from hPSCs

Bringing cell therapies to the clinic requires generation of differentiated end-stage cells that are safe, functional, and at a scale sufficient to replace damaged cells. Rezania et al. reported that their differentiation protocol generated ~26 million differentiated end-stage cells or ~13 million NKX6.1+ insulin+ β-like cells from a single T-150 flask of hPSCs [46]. Pagliuca et al. used a scalable suspension-based culture system to produce larger number of β-like cells [45]. In this study, 300 million differentiated end-stage cells were generated at a time in a single 500 mL spinner flask. They estimated that one or two flasks might be sufficient for treatment of a patient with diabetes. Considering that 7271 islet equivalents or 5.3 million β cells per kg of the body weight were transplanted to achieve insulin independence in a previous study [63], the number of β-like cells required for autologous cell therapy can be achieved by using current differentiation protocols.

Clinical applications of hPSCs

Historically, hPSCs have been used in clinical trials to treat patients with spinal cord injury, Parkinson's disease, ischemic heart failure, eye diseases, and type 1 diabetes [64]. These clinical trials include transplantation of differentiated cells to patients with the goal of potentially contributing to damaged tissue repair and treatment of disease. The first-in-human trial using hPSCs was initiated in 2010 by Geron Corporation [65]. Patients were injected with hESC-derived oligodendrocyte progenitor cells that have remyelinating and nerve growth stimulating properties. This study was followed by a dose escalation trial by Asterias Biotherapeutics, Inc. (ClinicalTrials.gov Identifier: NCT02302157). The preliminary findings seem promising as no serious adverse effects in the patients 12 months postinjection was reported and motor function improved in some cases [66]. The first-in-human trial using hiPSCs was conducted to treat one patient with age-related macular degeneration by injecting retinal pigment epithelium cells derived from autologous hiPSCs in 2014 [67]. One year after transplantation, the patient made progress and her vision stopped deteriorating [68]. For diabetes treatment, first clinical trial using hPSCs was initiated in 2014 by ViaCyte, Inc., as discussed earlier (NCT02239354). Allogeneic hESC-derived pancreatic progenitors were enclosed in retrievable delivery devices called Encaptra and implanted subcutaneously to 19 patients with type 1 diabetes. Preliminary findings demonstrated that the product was safe and well tolerated by patients with minor adverse effects. The engrafted cells showed positive staining for markers of islet cells such as insulin, NKX6.1, and glucagon. Although the devices appeared to protect the grafts from immune cell attack, graft cell survival was minimal in most cases at 12 weeks after implantation, which could be due to a development of foreign body response to the device leading to hypoxia [69]. Later on, in 2017, they initiated two clinical trials (NCT03162926 and NCT03163511) for testing a new open Encaptra device allowing direct vascularization of hESC-derived pancreatic progenitors [70]. They reported good tissue integration and vascularization of the system in two patients.

Protection from immune rejection

Immune rejection of transplanted cells remains a major challenge for the field. It is known that prolonged immunosuppressive treatment leads to dangerous side effects such as organ-system toxicity, infection, and cancer. To overcome this, researchers developed different strategies to protect cells from immune-mediated destruction. These strategies include (a) putting a physical barrier between therapeutic cells and immune cells by encapsulation, (b) autologous transplantation, and (c) generation of universal or hypoimmunogenic hiPSCs as discussed later (Fig. 14.3).

Encapsulation

The strategy of encapsulation of therapeutic cells in retrievable devices has promise for treatment of a variety of human diseases including autoimmune diabetes. While semipermeable barriers protect transplanted cells from the recipient's immune system, they allow release of therapeutic protein from grafts (insulin from hPSC-derived β-like cells in the case of diabetes). The major challenge faced in this strategy is insufficient oxygen supply to the encapsulated cells due to lack of tissue vascularization and development of fibrosis around the foreign encapsulation device material. To overcome these impediments, device design and materials are being developed and tested to provide sufficient oxygen to grafts and eventually maintain viability and function of encapsulated tissue [71].

Autologous transplantation

Alternatively, patient-specific hiPSCs could be used since transplantation of differentiated cells derived from these hiPSCs do not trigger allogeneic immune attack. Until now, hiPSCs have been derived from patients with diabetes including T1D (with or without complications) [72], T2D [73], and monogenic diabetes [74]. It has been demonstrated that hiPSCs derived from T1D patients were able to differentiate into pancreatic β-like cells [49]. Compared to β-like cells derived from non-T1D individuals, diabetic β-like cells demonstrated similar function and gene expression

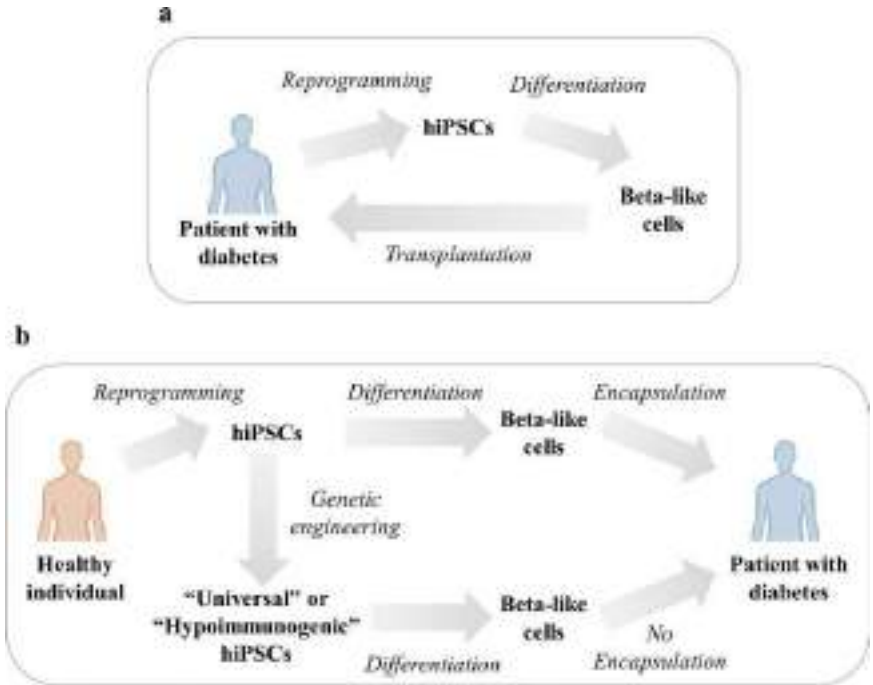

FIG. 14.3 Immune rejection of transplanted cells remains a major challenge for the field. To overcome this, different approaches are in consideration such as (A) autologous transplantation of β-like cells derived from patient-specific iPSCs, (B) encapsulation of hiPSC-derived β-like cells before transplantation to patient with diabetes, or generation of "universal" or "hypoimmunogenic" hiPSCs to transplant β-like cells to multiple patients without the need of encapsulation.

signature. Even though autologous transplantation is safe in terms of allogeneic rejection, differentiated cells would still be subject to autoimmune attack in the case of T1D. Another challenge is to generate and characterize clinical grade hiPSCs for individual patients which would take time and poses major logistical difficulties.

Generation of universal or hypoimmunogenic hiPSCs

To address the concern regarding generation of personalized hiPSC lines for each autologous transplantation, researchers have considered to create a bank of clinical grade hiPSC lines with the human leukocyte antigen (HLA) types matching the majority of individuals in a population [75]. Here, the challenge is to screen HLA types of thousands of people and to bank a few hundred of HLA-matched clinical grade hiPSCs. Since the immune barrier to cell therapy is a result of the mismatches of HLAs on the cell surface between recipient cells and donor cells, an alternative would be to develop hPSC products that do not express highly polymorphic HLA class Ia and class II molecules [76]. In addition to deletion of these HLA genes, insertion of immunomodulatory factors (PD-L1, HLA-G, and CD47) aims to protect hiPSCs against both adaptive and innate immune responses. Han et al. demonstrated that these gene-edited hiPSC products elicit significantly less immune activation, as well as less T-cell, NK-cell, and macrophage-mediated killing [77]. These universal or hypoimmunogenic hPSC products hold promise to be transplanted to multiple patients without the need of immunosuppressive drugs or encapsulation.

In summary, once overcoming the challenges reviewed here, stem cells provide an excellent tool for the treatment of patients with diabetes. Since limited donor availability widely restricts the use of islet transplantation and allows only a small number of patients benefit from this approach, stem cells including adult (MSCs), embryonic (hESCs), or induced (iPSCs) can instead be expanded, differentiated into disease relevant cells in vitro, and implemented to provide an unlimited source for cell therapies. There are significant challenges today to bring cell therapies to the clinic. Therefore, improvements in differentiation protocols to generate safe, functional stem cell products at large quantities, coupled with development of novel strategies to protect them from immune system attack, are promising for the future treatment of diabetes.

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PART IV

Causes of regenerative failure

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CHAPTER

15

Progression of kidney disease as a maladaptive response to injury

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Adapt or perish, now as ever, is nature's inexorable imperative.
H.G. Wells

The kidney is vulnerable to acute kidney injury (AKI) and has the regenerative capacity to repair itself after damage. This repair can make injured kidney return to normal morphology and function when the damage is minimal to mild. But when the response is more severe or is superimposed on some additional abnormalities, the adaptive repair process can become incomplete and even maladaptive, which can lead to renal fibrosis and eventually to chronic kidney disease (CKD) [1–3].

An organelle is a substructure that is directly related to various essential biological reactions within the cell, e.g., mitochondria, endoplasmic reticulum (ER), lysosomes, Golgi apparatus, and endosomes [4, 5]. Different organelles possess different functions, such as the production of energy, degradation of unfolded proteins. Organelles are emerging as determinants of cellular maladaptation to stress in acute or chronic kidney injury [6, 7]. The role of organelles in mediating maladaptive response in kidney injury are summarized in this chapter.

Mitochondria

Mitochondria are double-membrane organelles that generate adenosine triphosphate (ATP) by oxidative phosphorylation in highly metabolically active cells. Tubular epithelial cells require active transport to reabsorb most of the glomerular filtrate, so they have more mitochondrial content than other cell types in the kidney [7]. Surviving tubular cells after injury undergo dedifferentiation, proliferation, immigration, and redifferentiation into new tubular cells to repair the tubular injury. Mitochondrial dysfunction causes a decrease in ATP production and an increase in the production of reactive oxygen species (ROS), which affects various metabolic processes, including cell survival and death [8, 9]. Mitochondria have their own genome and perform certain biochemical functions, intracellular signaling, and dynamics required for cell survival and adaptation. Mitochondria are essential to programmed cell death, which is required for the removal of cells fatally damaged by stress that exceeds the cell's adaptive capacity. Mitochondrial structural alteration and dysfunction can lead to kidney cell injury and eventually progression of CKD [10]. In addition, mitochondrial dysfunction could occur even before detectable kidney injury. Funk et al. showed that mitochondrial dysfunction occurred before the increase of serum creatinine [11]. Brooks et al. further confirmed that mitochondrial fragmentation appeared before renal tubule cell apoptosis in a mouse model of ischemic AKI [12]. Therefore, maintenance of mitochondrial homeostasis is pivotal for the tubular cell regeneration after injury in the context of both AKI and CKD. Mitochondrial homeostasis requires a balance between mitochondrial biogenesis, fission, and fusion [13].

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Mitochondrial fusion and fission

Fusion and fission play a pivotal role in the quality control of mitochondria. Fusion and fission are complementary processes that are necessary for mitochondrial homeostasis. Fusion is the combination of two different mitochondria, while fission is the splitting of a mitochondrion into two daughter mitochondria. Fusion is controlled by mitofusin 1 (MFN1) and 2 (MFN2) on the outer membrane and activation of dynamin-like 120 kD (OPA1) on the inner membrane. Downregulation of MFN2, Parkin 1, and Parkin 2 or Ca^{2+} signaling may lead to renal inflammation and fibrosis [14]. Fission mediated through dynamin-related protein 1 (DRP1), which translocates from cytosol to the mitochondrial outer membrane and reacts with its receptor proteins including mitochondrial fission factor (Mff), fission-1 (Fis1), and mitochondrial kinetic proteins 49 and 51 (Mid49/51), and then DRP1 oligomerizes and cleaves the mitochondria (Fig. 15.1).

DRP1 reacts with mitochondrial fission 1 and then B-cell lymphoma 2 (BCL2)-associated X protein (BAX)/BCL2-antagonist/killer 1 (BAK)-mediated outer membrane permeabilization, which results in mitochondrial fragmentation and apoptosis. The translocation of DRP1 into the mitochondrial outer membrane occurs shortly after kidney injury [11, 12].

Fusion and fission support many mitochondrial functions. Fusion can be involved in mtDNA integrity, mitochondrial respiration, $\Delta\Psi m$ equilibration, apoptosis, and signaling events such as Ca^{2+} oscillations. Fusion redistributes mitochondrial proteins. Downstream of hyperfusion and following engagement of the mitochondrial E3 ubiquitin ligase/SUMOylase MULAN (also known as MAPL), NF-κB is activated, likely as an adaptive mechanism to promote antiapoptotic protein expression [15]. Moreover, hyperfused mitochondria can protect organelle from autophagic degradation. Fission can facilitate motility and is essential for the segregation of damaged mitochondria for mitophagy, mtDNA replication, and mitochondrial redistribution during cell division [16–20]. Fission is accelerated in AKI and CKD, such as diabetic nephropathy, it is harmful to mitochondrial homeostasis in the long term.

Mitochondrial biogenesis

DNA mutations, oxidative stress, substrate deprivation or oversupply, calcium and iron overload, cell death, toxins, viruses, and bacteria are stressors for mitochondria. Mitochondria can alter metabolic processes to adapt to stressors through a number of signaling pathways that maintain homeostasis. Mitochondria biogenesis (MB) can stabilize energy balance and limit oxidative damage. Mitochondria biogenesis is mainly regulated by peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) [10]. PGC-1α directly regulates many transcription factors involved in MB, including nuclear respiratory factor 1 (NRF-1), NRF-2, peroxisome proliferator-activated receptor alpha, estrogen-related receptor alpha, and YY1 transcription factor. Genetic deletion of *PPARGC1A* leads to downregulation of the genes related to oxidative phosphorylation, tricarboxylic acid (TCA) cycle, and glycolysis [21]. Deacetylation of PGC-1α allows its activation, and translocation to the nuclei, then upregulates the transcription of target genes that regulate mitochondrial homeostasis and ATP production [22]. PGC-1α is persistently suppressed during AKI, and the restoration of PGC-1α to basal levels is necessary for renal function recovery. *PPARGC1A* l-knockout mice show worse renal function in response to sepsis [10, 11]. And decreased levels of PGC-1α have also been observed in CKD, like diabetic nephropathy [23].

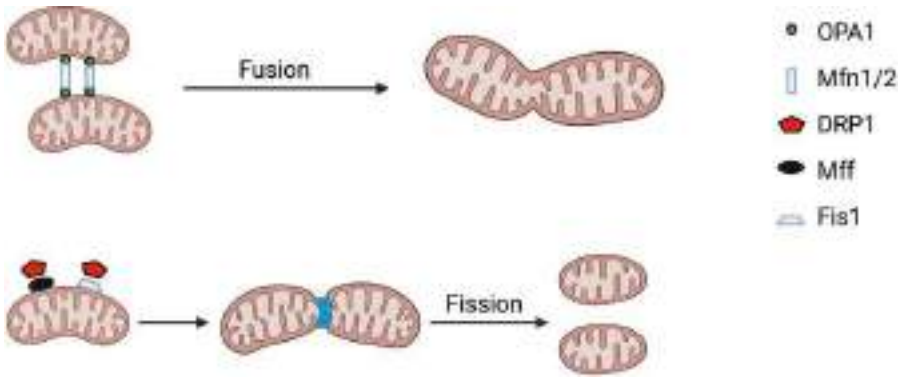

FIG. 15.1 Mitochondrial fusion and fission.

On the contrary, overexpression of PGC-1α in cultured renal proximal tubule cells ameliorate oxidant exposure [24]. Increasing the level of PGC-1α can upregulate MB in the recovery following IRI [25, 26].

Endoplasmic reticulum (ER) stress

ER is an important organelle involved in maintaining the quality of proteins through the unfolded protein response (UPR). ER is very sensitive to alterations of intracellular homeostasis. ER stress is a response to the accumulation of unfolded or misfolded proteins in the ER lumen. External stimuli, such as nutrient excess/deprivation, oxidative stress, iron imbalance, Ca^{2+} leakage from ER, viral infections, and hypoxia, results in the accumulation of unfolded proteins, which is a direct cause of ER stress. ER stress has been described in AKI, glomerulonephritis, hypertension-associated kidney disease, and diabetic kidney disease. ER stress is mainly regulated by three major ER stress sensors, including protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring protein 1 (IRE1), and activating transcription factor 6 (ATF6). These sensors are ER transmembrane proteins with an ER luminal domain which can detect the unfolded or misfolded proteins. Under physiological conditions, the ER stress sensor proteins bind with ER chaperone protein, such as glucose-regulated proteins 78 (GRP78, also known as BiP), to maintain an inactive state. When under stress, ER stress sensor proteins dissociate from chaperone protein and active downstream transcription factors to promote the degradation of unfolded or misfolded proteins through ER-associated protein degradation (ERAD) or autophagy (Fig. 15.2).

ER stress sensor

PERK-eukaryotic initiation factor 2 α (eIF2α)-ATF4 pathway

Activation of PERK is the most immediate response in UPR. Activated PERK phosphorylates eIF2α on Ser51 and reduce general protein synthesis to limit protein-folding load into ER, and activate transcription of UPR survival

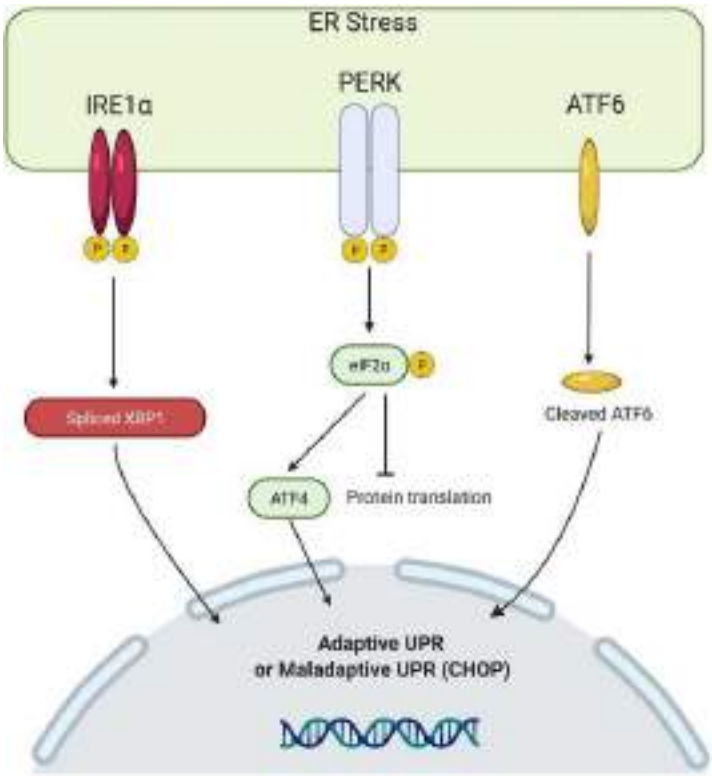

FIG. 15.2 The unfolded protein response (UPR) pathway in the endoplasmic reticulum (ER) stress.

genes downstream of eIF2α. Moreover, PERK can also activate the transcription factor ATF4, which sequentially induces gene encoding amino acid biosynthesis and transport functions, antioxidative stress responses, and CCAAT-enhancer-binding protein homologous protein (CHOP)/GADD153 and proapoptotic BH3-only BCL2 family members NOXA and BIM, caspase3/7, leading to apoptosis [27].

IRE1-X-box-binding protein 1 (XBP1) pathway

IRE1 is activated through homodimerization and transautophosphorylation, and then also leads to transcriptional activation of CHOP. The IRE1 genome contains two homologs, IRE1α and IRE1β. Activated IRE1α can function as an endoribonuclease to remove a 26-nucleotide intron from XBP1 mRNA. While spliced Xbp1 mRNA induces expression of enzymes that help protein folding, or degradation. IRE1α can recruit TNF receptor-associated factor 2 (TRAF2) and c-Jun-N-terminal inhibitory kinase (JIK) to ER membrane under ER stress. TRAF2 actives apoptosis-signaling kinase 1 (ASK1), which leads to mitochondria-dependent caspase activation.

ATF6 pathway

ATF6 is a bZIP transcription factor of CREB/ATF family, with two forms (ATFα and ATFβ). Under ER stress condition, ATF6 is transported to Golgi complex and cleaved by site-1 protease (S1P) and site-2 protease (S2P) to form a 50-kD cytosolic bZIP-containing fragment which migrates into the nucleus to dock to ER-associated binding elements to activate UPR target genes.

Maladaptive UPR response in CKD

Transient activation of UPR can trigger prosurvival pathways to restore the balance between misfolded and folded protein in the early stage of UPR activation. Conversely, UPR also can trigger proapoptotic pathways if activation becomes sustained, and maladaptive, and finally lead to ER dysfunction. The persistent ER dysfunction induced renal tubular cell apoptosis, inflammation, and autophagy. Shu et al. reported a sustained activation of ER stress in AKI-CKD transition by using a unilateral renal ischemia-reperfusion injury mice model. And pharmacological inhibition of ER stress after AKI reduced expression of BiP and CHOP, attenuated renal tubular cell death, inflammation, autophagy, and fibrosis and renal function was improved in post ischemia-reperfusion kidneys [28].

Apoptosis

ER stress can initiate apoptosis through direct ER-mitochondrial interactions. ER is the major intracellular calcium reservoir in the cell, both protein-folding reactions and protein chaperone functions require high levels of ER intraluminal calcium. And numerous ER chaperones act as calcium-binding proteins. Uptake of calcium by mitochondria is highly regulated by ER membrane-resident sarcoendoplasmic reticulum Ca^{2+}/ATPase (SERCA) pump. Impairment of SERCA increases calcium leakage, enhances apoptotic susceptibility of cells [29–31].

In addition, chronic ER stress leads to BAX/BAK-dependent apoptosis through upregulation of BCL-2-interacting mediator of cell death (BIM) and p53 upregulated modulator of apoptosis (PUMA) [32]. Chronic ER stress and sustained IRE1 activation triggers the PERK-eIF2α-CHOP-Caspase 3/7 proapoptotic cascade and result in reduced XBP-1 splicing and IRE1 turns to an important executor of apoptosis [33, 34].

Our group has identified a new ER-associated protein reticulon 1 (RTN 1), specifically, the isoform A (RTN1A), which can induce apoptosis of renal epithelial cells through PERK and CHOP activation in models of AKI to CKD transition, UUO, and DKD [35–37]. We found that RTN1A expression is increased significantly in the tubulointerstitial compartment of the diseased kidney and correlated with the decline of renal function. Several RTN1A variants were also identified in diabetic patients with ESRD. In vitro, overexpression of RTN1A induces ER stress and apoptosis of renal tubular epithelial cells. In vivo, overexpression of RTN1A induces more severe kidney injury with worsening of renal function. On the other hand, knockdown of RTN1a in renal tubular cells attenuates kidney injury in multiple kidney disease models such as diabetic nephropathy, HIV nephropathy, and AKI to CKD transition. Therefore, we believe that RTN1A could be a potential therapeutic target for CKD progression.

Chronic inflammation

Chronic activation of ER stress sensor can result in the activation of proinflammatory transcription factors. IRE1α stimulates tumor necrosis factor receptor-associated factor and AP-1 through IRE1 α-mediated TRAF2 signaling [38],

and TRAF2 promotes NF-κB, NOD1/2, and RIP-dependent cascades [39–41]. Similarly, PERK activation can increase NF-κB stability through the inhibition of I-κB [42]. ER stress is also characterized by an increase in proinflammatory cytokines, such as IL-1β, IL-6, and IL-18 [43].

In addition, ER stress can alter the phenotype of inflammatory cells, particularly macrophages. Genetic deletion of IRE1α and CHOP in macrophages limited M1 polarization and increased the alternatively activated (M2) macrophage phenotype [44, 45]. And ER stress can generate the M2 phenotype differentiation in mouse peritoneal macrophages through JNK activation and increased PPARγ expression [46]. Nogo-B, an ER-associated protein and a family member of reticulon, can regulate the recruitment of macrophages in unilateral ureteral obstruction (UUO) [47].

Fibrosis

Tubular epithelial cells undergoing sustained ER stress and UPR activation can transdifferentiate into myofibroblast cells. During this course, epithelial cells adopt mesenchymal cell-like morphology and increased expression of vimentin, N-cadherin, and α-smooth muscle actin (α-SMA). Acquisition of mesenchymal phenotype can secrete profibrotic growth factors, such as TGF-β [48], and drive the renal fibrotic process [49–51].

Crosstalk between ER and mitochondria in maladaptive response in kidney injury

Adaptive UPR helps to maintain ER homeostasis, it also regulates dynamic interactions between ER and mitochondria [52]. As increasing UPR consumes a huge amount of ATP, cells increase the contact area (mitochondria-associated membranes, MAMs) between ER and mitochondria, which in turn accelerate the release of calcium from ER to mitochondria. An appropriate increase of calcium concentration activates the tricarboxylic acid cycle dehydrogenase to promote ATP production. If the UPR cannot reduce cell stress, severe ER stress leads to mitochondria calcium overload, mitochondrial dysfunction, activation of proapoptotic factors, and finally lead to apoptosis [53, 54] (Fig. 15.3).

MAMs can activate the NOD-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome, which subsequently induces infection defense via inflammatory cytokines, such as interleukin-1β/interleukin-18. NLRP3 was reported to contribute to nephrocalcinosis-related CKD, diabetic kidney disease, and gout [55, 56] (Fig. 15.3).

Nogo-B is an ER protein, can be activated by ATF6. Nogo-B increases the distance between ER and mitochondria and decreases ER to mitochondria phospholipid transfer and intramitochondrial calcium [57]. Our recent unpublished data suggest that RTN1A also involves ER-mitochondrial crosstalk leading to more tubular cell apoptosis. ATF4 is an important regulator of autophagy. Eif2α/ATF4 regulates autophagy gene transcription in response to amino acid starvation or ER stress [58].

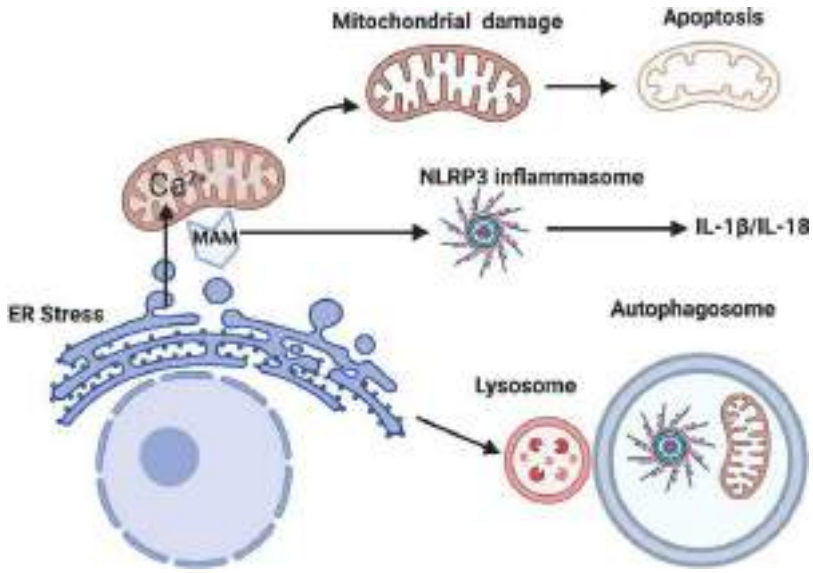

FIG. 15.3 Crosstalk between endoplasmic reticulum and mitochondria.

Lysosomes and autophagy

The lysosome is a membrane-bound organelle, contains hydrolytic enzymes, and acts as a digestive system in cells. The function of the lysosome is fulfilled by the mechanism called autophagy. Damaged proteins, polysaccharides, and dysfunctional organelles are engulfed by autophagosomes, then deliver to and fuse with lysosomes, digested by protease, resulting in the production of amino acids and monosaccharides [59, 60]. mTORC1 is the key regulator of autophagy and lysosome stress response. Autophagy involves initiation, nucleation, expansion, fusion, and degradation. When cells are starved, or under high levels of amino acids, mTORC1 is recruited to the lysosomal membrane and activated. Activated mTORC1 phosphorylates ribosomal protein S6 kinase (S6K), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), the autophagy regulator ULK1, 2, and the transcription factor TFEB [61, 62]. Moreover, the class III phosphoinositide 3-kinase (PI3K) complex regulates vesicle nucleation. The autophagy-associated protein (ATG)12-ATG5-ATG16L system and microtubule-associated protein 1 light chain 3 (LC3) system control the expansion and completion of the autophagosome [63, 64].

A growing body of evidence has suggested that autophagy plays an important role in the pathogenesis of CKD. A basal level of autophagy in resident kidney cells, such as podocytes, mesangial cells, and glomerular endothelial cells, and proximal tubule epithelial cells (PTECs) is necessary for maintaining normal cellular physiology. In vitro studies suggest that autophagy regulates the turnover of collagen and has cytoprotective role in response to serum starving [65, 66]. In AKI, intracellular stress in PTECs stimulates autophagy to enhance phagocytosis of damaged cells and inhibits inflammatory response. While persistent autophagy stimulates the secretion of profibrotic cytokines, and leads to tubulointerstitial fibrosis. Inhibition of mTOR in PTECs after ischemic AKI leads to impaired PTECs proliferation, and tightly regulated autophagy is crucial for PTECs regeneration [67]. Persistent autophagy in maladaptive response in kidney repair involves complex mechanisms, Atg5, Atg7, Foxo3, HIF1α, G2/M cell cycle arrest, telomerase, and ER stress all involved in tubular senescence and interstitial fibrosis in AKI to CKD transition [68–71].

Mitophagy is a selective degradation pathway of mitochondria following damage or stress by autophagy, which can protect stressed cells from death. Mitophagy mediators include phosphatase and tensin homolog-induced kinase 1 (PINK 1) and Parkin (E3 ubiquitin ligase), FUN14 domain-containing protein 1, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3, and BNIP3-like protein. Loss of mitochondrial membrane potential is a signal for mitophagy [18]. Activation of mitophagy in renal tubular cells has a protective role during AKI. Hypoxia can trigger mitophagy by activation of BCL2/BNIP3 and BNIP3L through HIF-1α. NLRP3 negatively regulated mitophagy in renal tubular cells through an inflammasome-independent manner under hypoxia [72]. In NLRP3 knockout mice, unilateral ureter obstruction accelerated mitophagy in renal tubular cells. PINK1-MFN2-PARK2 pathway deficiency in macrophages leads to accumulation of abnormal mitochondria, an increase of ROS, and upregulation of RPTOR-independent companion of MTOR complex 2, and promoted differentiation toward M2 (profibrotic) phenotype in UUO mice [14]. These recent findings suggest that maladaptive regulation of mitophagy involved in the pathogenesis in renal fibrosis.

Conclusion

Organelles stress and their crosstalk play an important role in the maladaptive response in various kidney injury. It is important for us to further elucidate how organelle stress is evoked in the context of kidney cell injury. An elucidation of these mechanisms may help us to identify potential new therapeutic targets for both AKI and CKD.

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CHAPTER

16

Molecular mechanisms of cellular senescence

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What is cellular senescence and why it is important?

Cellular senescence is characterized by a stable cell cycle arrest accompanied by a hypersecretory phenotype, macromolecular damage, altered metabolism, and structural changes [1]. The senescence response is traditionally considered a tumor suppressor mechanism as it limits the proliferation of unwanted and/or damaged cells [2]. In the last two decades, numerous studies demonstrated the importance of cellular senescence in many aspects of tissue physiology and pathology, emphasizing that a key determinant for the beneficial versus detrimental functions of senescent cells might be their temporal persistence in tissues (Fig. 1). Strikingly, cellular senescence seems to be an important driver of aging and many age-related pathologies [3]. How cellular senescence drives aging phenotypes is not completely elucidated, but it is thought to be the combination of cell autonomous and noncell autonomous mechanisms. Senescent stem and progenitor cells reduce the regenerative capacity of a tissue, while the secretion of a complex combination of factors, collectively known as the senescence-associated secretory phenotype (SASP), influences the function of neighboring cells. Although chronic senescence appears detrimental, transient and localized senescent cells can have beneficial properties. For example, controlled and transient induction of senescent cells and their SASP can promote regeneration and limit fibrosis upon injury, increase tissue plasticity and reprogramming in different organs, and ensure accurate tissue patterning during embryogenesis [4] (Fig. 1).

Discovery

In 1912, Alexis Carrel established the first "cell line" derived from the heart of a chicken embryo. These cells were cultured until Carrel died in 1946 [5]. The observed continuous and unrestrained proliferation established the dogma that cells are intrinsically immortal and that the failure to maintain indefinite cell growth in previous attempts was simply due to inadequate culture conditions [5]. Fifty years later, Leonard Hayflick and Paul Moorhead disproved Carrel's theory of "cellular immortality" by demonstrating that primary human cells can undergo a limited amount of cellular divisions [6]. This finite replicative life span of cells, defined as cellular senescence, was postulated to be the cellular reflection of organismal aging [6, 7], and was initially shown to be the consequence of telomere attrition, also known as replicative senescence (RS) [8, 9]. Following studies have then demonstrated that cells can undergo senescence in response to various types of stressor, a phenomenon generally defined as stress-induced premature senescence (SIPS).

Triggers of cellular senescence

DNA damage, telomeres, and oxidative stress in cellular senescence

One of the most recognized triggers and hallmarks of cellular senescence is unresolved DNA damage, mostly in the form of double-strand breaks (DSBs) [10]. Extrinsic agents, such as ionizing and nonionizing radiation and chemotherapeutic drugs, and intrinsic factors, such as telomere attrition and oxidative, oncogenic, or replicative

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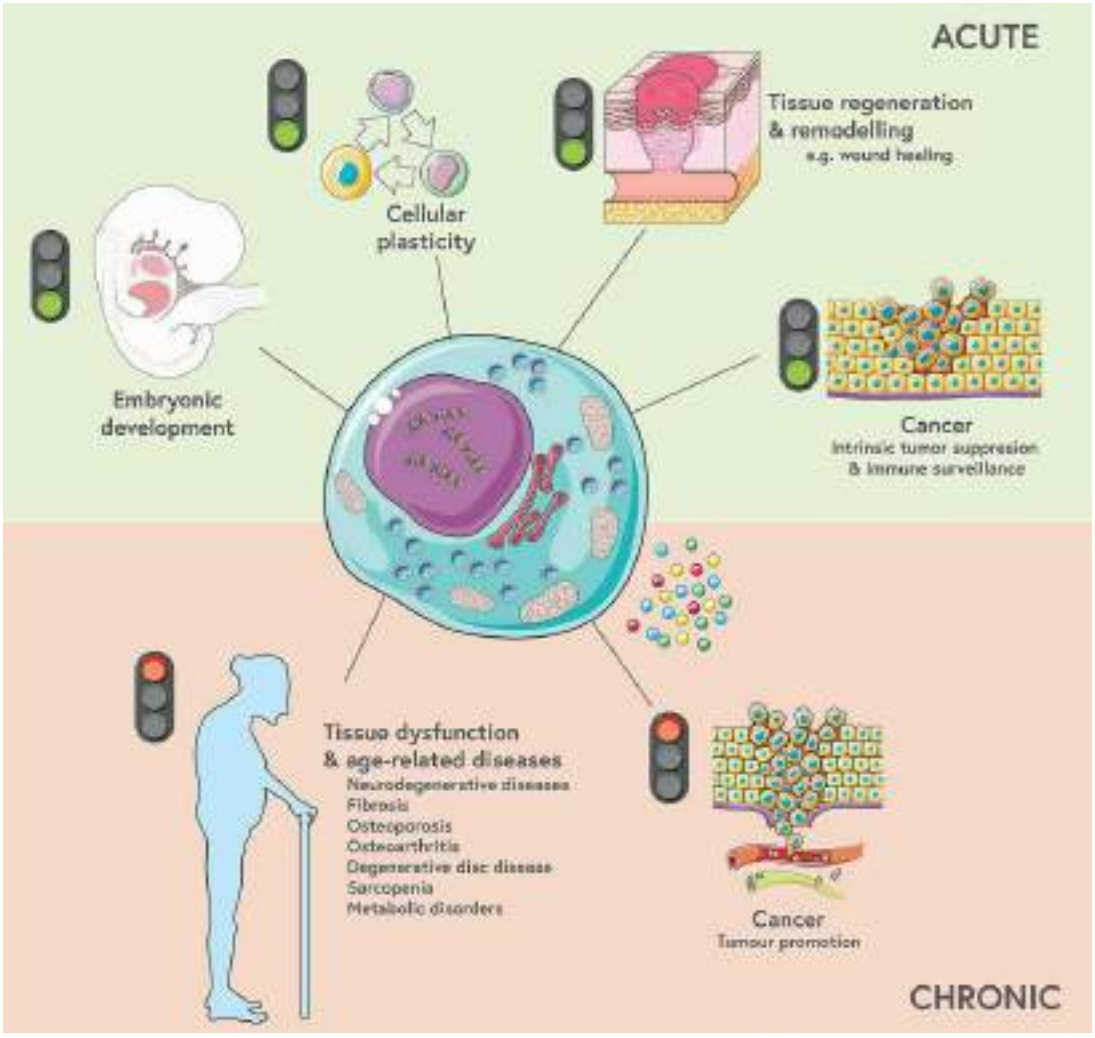

FIG. 1 Biological functions of cellular senescence. Acute or transient senescence exhibits a beneficial role in tumor suppression, tissue regeneration, cellular plasticity, and embryonic development. Chronic or persistent senescence promotes age-related pathologies, including cancer.

stress, can promote senescence via DNA damage. DNA lesions are sensed by protein complexes that activate two large kinases: ataxia telangiectasia and Rad3 related (ATR) and/or ataxia telangiectasia mutated (ATM). Activation of ATM and ATR can diffuse the signal beyond the lesion site through the activation of checkpoint kinases CHK1 and CHK2, which ultimately lead to cell cycle arrest via the p53/p21 axis [11]. Although damage occurring in any genomic region can eventually promote senescence, damage at telomeric sites might play a predominant role [12].

Telomeres are nucleoprotein structures consisting of a repetitive sequence of DNA (TTAGGG) associated with numerous proteins part of the Shelterin complex. Telomeres are found at the end of linear chromosomes of eukaryotic cells where they form a protective cap against the recognition of chromosomal ends as double-strand DNA breaks, which could otherwise result in activation of DNA repair mechanisms leading to extensive genomic instability [13]. Telomeres shorten with each cell division due to the inability of DNA polymerases to completely replicate the lagging strand during DNA replication, a phenomenon termed "end-replication problem" [14]. This progressive loss of telomeric repeats expose telomeric ends to the recognition from the DNA repair machinery leading to the activation of a DNA damage response (DDR) [15].

Although cell division being a major contributor to telomere loss, additional factors were shown to affect the rate of telomere shortening. For example, it has been shown that replicative potential and rate of telomere shortening correlate with the cellular antioxidant capacity [16]. Overexpression of the antioxidant enzyme superoxide dismutase in fibroblasts can reduce telomere shortening and increase replicative life span [17]. It is thought that telomere shortening, under mild oxidative stress, results from the accumulation of single-strand DNA damage that prevents the

complete replication of the chromosome during cellular division. Consequently, the sequence beyond the damage will be lost and telomere shortening accelerated [18].

Because guanine-rich sequences are highly susceptible to oxidative stress, telomeres are believed to be a favored targets of reactive oxygen species (ROS)-induced damage compared to the rest of the genome [19]. Moreover, it has been shown that telomeric lesions can remain unrepaired for an extended period of time irrespectively of telomere length suggesting that persistent telomeric damage response might be an important factor for the establishment of the senescence phenotype [20].

Developmental or programmed senescence

While sporadic and acute DNA damage represents a key senescence inducer, recent studies have also emphasized the importance of physiologically controlled triggers [21]. During embryonic development, induction of cellular senescence appears to be spatially and locally restricted, and to occur identically in every embryo of the same species, suggesting the existence of an intrinsic program [4]. The fact that programmed senescence has been detected in many different species proposes that senescence might have originally evolved as a mechanism for tissue remodeling and then acquired additional roles throughout evolution. Some identified developmental cues responsible for the induction of developmental senescence involve the PI3K/FOXO, TGFβ/SMAD, and ERK pathways [22]. These pathways seem to induce senescence by upregulating the cell cycle inhibitor p21 without any obvious DNA damage signal [22].

Cell cycle arrest

Cell cycle is a highly conserved and precisely coordinated process, necessary for cell growth and division, which consist of four phases denominated as gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M). Cell cycle is mainly regulated by complexes formed by cyclins and cyclin-dependent kinases (CDKs), which control cell cycle progression through the phosphorylation of target genes [23].

The existence of cell cycle checkpoints allows for a strict control of cell division [23]. Stable cell cycle arrest is one of the main features of cell senescence and, unlike quiescent cells, senescent cells are unable to reenter cell cycle even when environmental conditions are set for cell proliferation.

During cell cycle regulation, CDK4/6-cyclin D complexes promote G0-G1 cell cycle progression, while CDK2-cyclin E complexes are responsible for promoting the G1-S phase. Senescent cell cycle arrest is predominately characterized by the activation of the CDK inhibitors (CDKi) p21 and p16 networks, which mainly block CDK-cyclin complexes involved in the G0/G1-S cell cycle checkpoints [24]. p21 is consistently upregulated as consequence of different stressors. The oncosuppressor p53 induces the transcription of p21, blocking CDK2-cyclin E activity and leading to hypophosphorylation of the retinoblastoma protein (Rb), inhibiting the activation of the E2F genes, and resulting in cell cycle exit. However, it is worth mentioning that p21 can also be activated in a p53-independent manner [25]. p16, currently recognized as one of the most specific senescence markers, directly interacts and inhibits the CDK4/6-cyclin D complex which, similarly as it occurs with p21, blocks Rb phosphorylation and inhibits the activity of E2F proteins leading to a durable arrest. P16 can be activated by persistent stress stimulus, and epigenetic changes were suggested to be main inducers of its upregulation [26, 27].

Morphological and functional changes

Cell cycle-arrested senescent cells continue to receive growth-stimulatory factors, resulting in dramatic alterations of cell morphology and organelle composition, and consequent functional and structural changes (Fig. 2). In culture, senescent cells are typically enlarged, flattened, and vacuolated, and sometimes multinucleated or displaying enlarged nuclei [10, 27, 28]. The altered morphology is more difficult to identify in vivo, as enlargement and resizing might be restricted by the tissue architecture. Altered cell shape arises as a consequence of cytoskeleton rearrangement [29], which not only contributes to the aberrant morphology, but also reflects in functional changes involving intracellular trafficking, motility, polarity, and cell division [30, 31]. The intermediate filament protein vimentin seems to be upregulated and rearranged in senescence [32], together with increased abundance and reorganization of microtubules [29].

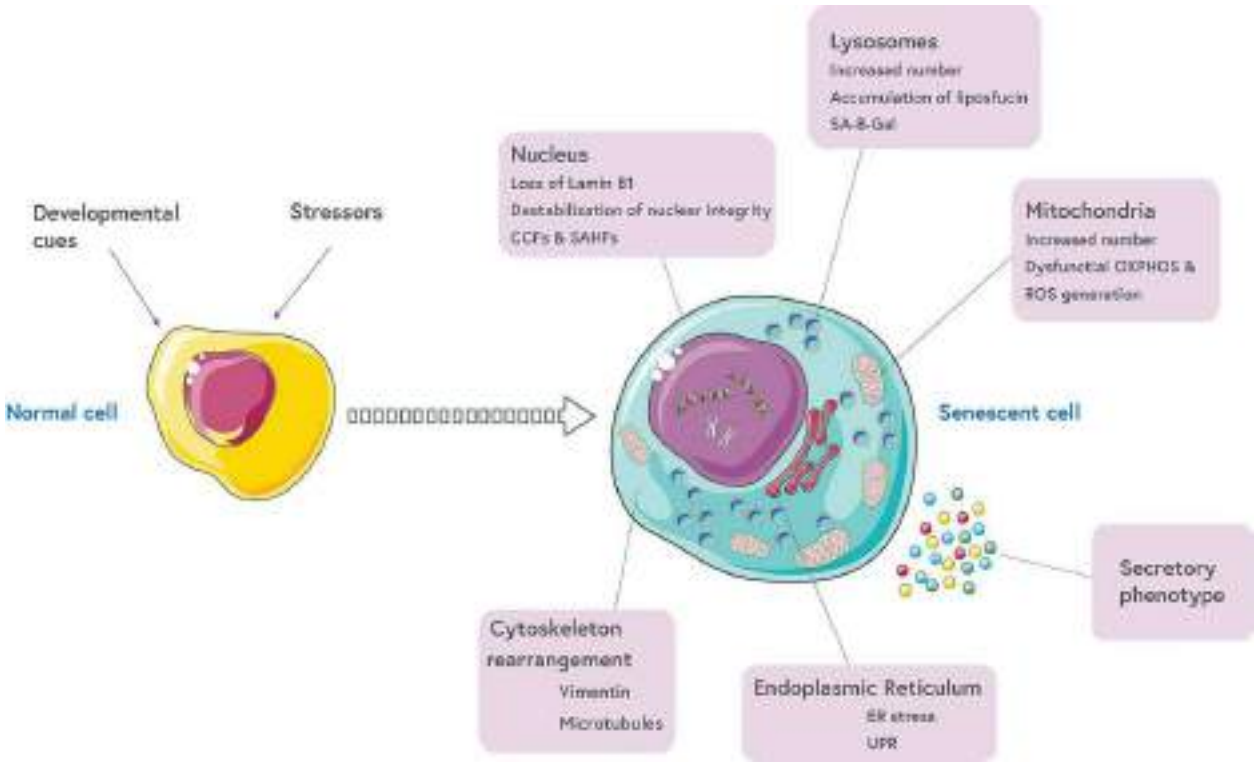

FIG. 2 Senescence-associated phenotypes. Senescent cells present different morphological, structural, and functional alterations that can serve as biomarkers for their identification or target for their elimination.

Because of the increased protein synthesis demanded by the SASP, senescent cells suffer from endoplasmic reticulum (ER) stress and consequent activation of the unfolded protein response (UPR) [33]. As a result, enlarged ER can be detected in senescent cells, sometimes characterized by disorganized cisternae [34].

In parallel, senescent cells display an increased lysosomal content due to increased biogenesis and reduced removal of old lysosomes. The elevated activity of the lysosomal enzyme SA-β-galactosidase, encoded by the *GLB1* gene, is a reflection of the increased lysosomal mass observed in senescent cells [35]. Lipofuscin-loaded lysosomes can also be observed in senescent cells and stained with specific dyes [36].

Even though senescent cells display a higher number of mitochondria, partly due to reduced mitophagy, mitochondrial dysfunction is an additional characteristic of senescent cells [37]. Deterioration of the mitochondrial OXPHOS, a complex sequential reaction necessary for producing and maintaining the energy in the cell, has been shown to be involved in early stages in senescence [38, 39]. Dysfunctional OXPHOS generates ROS, which then can directly damage DNA and other cellular components. In fact, mtDNA is highly vulnerable to ROS and leads to defective OXPHOS reaction and continuously increasing ROS production [40, 41]. Other mitochondrial alterations, such as highly interconnected or elongated mitochondria, are also typically observed in senescent cells and contribute to dysfunctional OXPHOS [41]. In addition, these mitochondria overproduce retrograde signaling messengers (ROS, calcium, NAD^+, AMP), which can modulate the activity of different proteins such as mTOR [42].

The loss of the lamina protein Lamin B1 participates in functional and structural changes of the nucleus during senescence. It is downregulated at different levels in senescent cells in a p53/p16-dependent manner. This results in destabilization of the nuclear integrity and is accompanied by other nuclear changes such as the appearance of cytoplasmic chromatin fragments (CCFs) and the loss of condensation of constitutive heterochromatin [43, 44].

The SASP

Even though the notion that senescent cells change their environment by secreting different factors dates back to the 1980s, the term SASP was only coined in 2008 [45]. Three pioneering studies described the SASP as a transcriptionally controlled phenotype that, despite some variability of its composition between different cell strains, commonly encompass inflammatory and tumorigenic elements [45–47]. Ever since its formal description, numerous

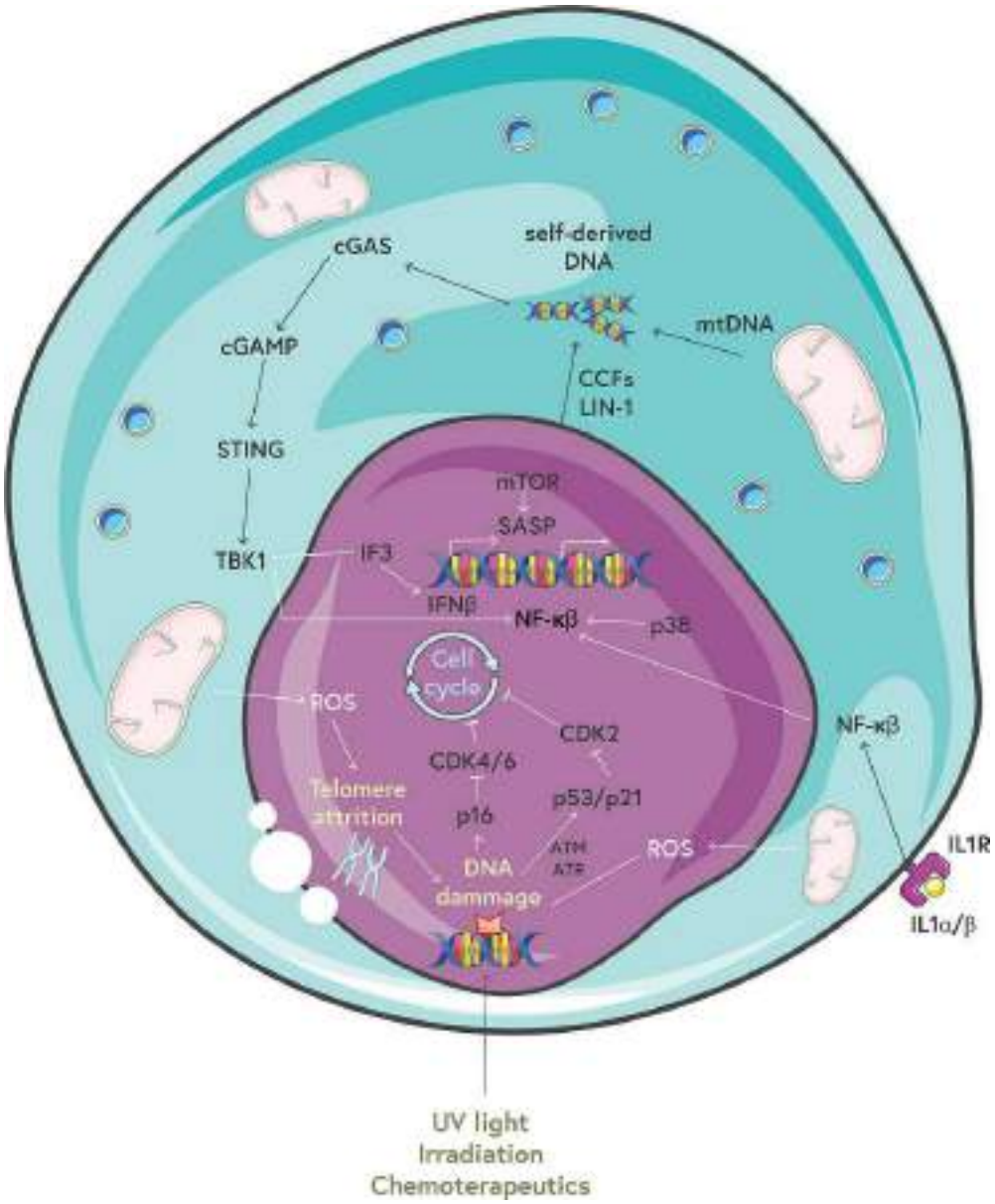

FIG. 3 Mechanisms controlling cell cycle arrest and the SASP. Multiple stressors activate cell cycle withdrawal through two major pathways, the p16INK4a/Rb and the p53/p21CIP1. The SASP is activated by the transcription factors NF-κB and C/EBPβ, which serve as converging point of various regulatory pathways, and by the interferon response, which is triggered by endogenous cytoplasmic DNA.

studies have discovered a multitude of pathways and triggers able to establish and maintain the various arms of the SASP (Fig. 3). For this reason, the SASP is now seen as a highly heterogeneous and context-dependent phenomenon with pleiotropic biological functions.

Components of SASP

As mentioned earlier, the composition of the SASP is not discrete, and many of its constituents are expressed only by subsets of senescent cells and under certain conditions. Factors influencing this heterogeneity are both intrinsic and extrinsic and include cell and tissue of origin, stressors, and surrounding environment. Generally, the SASP encompasses a panel of inflammatory, growth, and proteolytic factors that can influence the immediate microenvironment. The SASP can act both autocrinally, by reinforcing the senescence state [46–48], and paracrinally, by modulating the behavior and function of neighboring cells, including the induction of surrounding tissue into senescence (paracrine senescence) [49–51].

Numerous identified SASP constituents are receptor-binding molecules which can activate intracellular signaling cascades involved in inflammation and growth: interleukins (e.g., IL-1α, − 1β, − 6, − 7, and − 8), chemokines (e.g., CCL-2, − 5, and − 16), and growth factors (e.g., epidermal, fibroblast, hepatocyte, and keratinocyte growth factors). SASP also incorporates direct-acting molecules that specialize in the remodeling and degradation of the extracellular matrix, in the solubilization of membrane-bound receptors, and in the proteolytic cleavage of signaling molecules. This primarily includes a range of matrix metalloproteases (e.g., MMP-1, − 3, − 9, − 10, − 12, − 13, and − 14), serine proteases (e.g., tissue and urokinase plasminogen activators), and nonprotein components (e.g., nitric oxide and reactive oxygen species). Additionally, SASP may integrate a number of regulatory factors that can bind to and modulate the activity of constituents belonging to the two aforementioned categories. Such factors include tissue inhibitors of metalloproteinases (TIMPs and ADAMs), and plasminogen activator inhibitors (PAI) [52].

SASP induction and regulation

Multiple effectors appear to control the SASP, and particular effort has been spent in identifying regulators of the inflammatory arm. The DDR triggers an early form of SASP, which amplifies over time in an autocrine manner to produce a mature SASP [53]. Among the factors involved in DDR, ATM, Nibrin, and CHK2 were shown to be required for the induction of an early SASP [54]. ATM, in addition to ATR, can stabilize the transcription factor GATA4 and prevent its selective autophagy by p62. Stabilized GATA4 enhances the transcription of inflammatory genes facilitating IL-1α-induced NF-κB activation [55].

Another essential pathway activated in response to stress is the mitogen-activated protein kinase p38 (p38MAPK) pathway. It has been demonstrated that p38MAPK inhibition prevents the transcription of several SASP factors, independently of DDR, and that its constitutive activation is sufficient to induce part of the SASP [56, 57]. p38MAPK might regulate the production of SASP factors via the mitogen- and stress-activated kinases (MSK) 1 and 2. It has been reported that MSK 1 and 2 can potentiate inflammatory responses through the phosphorylation of either of the transcription factors CREB and STAT3 [58] or the p65 subunit of NF-κB [59]. Additionally, it was shown that p38MAPK can enhance the accessibility of NF-κB complexes to certain NF-κB-dependent promoters of genes involved in inflammation through the phosphorylation of the histone protein H3 [60].

IL-1α and IL-1β are prominent early-phase regulators of the SASP. Recently, it was identified that both cytokines, through interactions with their receptor IL-1R, are upstream regulators of proinflammatory SASP factors, but they are dispensable for the senescence-induced cell cycle arrest [61]. It has been reported earlier that the IL-1 pathway acts as an upstream regulator that can drive the autocrine forward loop that amplifies the SASP [62]. The IL-1 signaling cascade triggers the nuclear translocation of NF-κB and subsequently the transcription of different highly secreted SASP factors. The activation of NF-κB creates a positive feedback loop where NF-κB enhances the transcription of both IL-1α and IL-1β, thus leading to SASP amplification [63].

Another crucial player for regulating the SASP is cytoplasmic DNA [64]. In normal cells, self-DNA is localized within either the nucleus or the mitochondria to prevent activation of the proinflammatory cellular machinery that detects cytoplasmic DNA. However, cellular stress and DNA damage can facilitate the translocation of nuclear DNA, and the expulsion of mitochondrial DNA into the cytoplasm [64]. Additionally, the reduced expression of the nuclear lamina protein LMNB1 leads to the disintegration of the nuclear envelope and the extrusion of chromatin-containing fragments of nuclear membrane into the cytoplasm, forming cytoplasmic chromatin fragments (CCFs) [65]. It is therefore suggested that self-derived DNA (i.e., CCFs and mtDNA) could be sensed by the antiviral DNA sensor cGAS. Activated cGAS then synthesizes the secondary messenger cGAMP, which in turn activates STING pathway, leading to the autophosphorylation of TBK1. Activated TBK1 can induce the transcription of inflammatory cytokines by activating the NF-κB pathway or by phosphorylating IRF3, ultimately inducing type I interferon response [64]. STING activation is also correlated with the expression of IL-1α which can, as mentioned earlier, drive an autocrine forward loop that amplifies SASP production [62].

Typically, single- and double-stranded cytoplasmic DNAs are cleared by DNases. However, expression of cellular DNases such as DNase2 and TREX1 is downregulated in senescent cells, a mechanism that could explain the accumulation of cytoplasmic DNA [66]. This reduction is mediated by a p16-induced inactivation of the transcription factor E2F, which is needed for the expression of DNase2 and TREX1. This event, in synergy with the senescence-associated expulsion of nuclear DNA, may facilitate the expression of a plethora of inflammatory cytokines involved in SASP through cGAS-STING-mediated NF-κB signaling.

Most of the mentioned SASP regulatory mechanisms preferentially control the proinflammatory arm, which is highly conserved among different studied forms of senescence. These pathways regulating the transcriptional initiation and maintenance of SASP components converge on the two major transcription factors NF-κB and C/EBPβ.

It is suggested that C/EBPβ synergizes with NF-κB to trigger the transcription of prominent SASP factors. These transcription factors were shown to be activated and enriched in the chromatin fraction in senescent cells [46, 47]. It is worth mentioning that these transcription factors dominate the second or mature phase of the SASP, whereas the first or early senescent response is orchestrated by Notch, which inhibits the transcriptional activity of NF-κB and C/EBPβ and promotes the secretion of TGFβ [67].

Besides transcriptional regulation, SASP production is controlled by additional mechanisms, including mRNA stability, translation, and secretion. For example, mTOR inhibition or depletion prevents the expression of certain SASP components in senescent cells without affecting the growth arrest [68, 69]. On one side, mTOR regulates the translation of MAPKAPK2 through the initiation factor 4E-BP1 during senescence. Once activated, MAPKAPK2 then phosphorylate and inhibit ZFP36L1, an mRNA-binding protein that targets some SASP components for mRNA degradation. Therefore, mTOR inhibition leads to downregulation of MAPKAPK2 to levels that are insufficient to block ZFP36L1, ultimately leading to the degradation of mRNAs of various SASP components, including various proinflammatory cytokines [68]. On the other side, mTOR inhibition reduces NF-κB activity through blockade of the IL-1α/NF-κB forward loop. It was shown that rapamycin reduced the protein levels of the surface-bound IL-1α in senescent cells by negatively affecting its translational efficiency. This results in a reduced binding of the IL-1α cell-surface receptor IL1R1 onto cells, which subsequently reduces the degradation of both IRAK1 and IkBa, NF-κB activation, and thereafter SASP production [69].

Prosurvival mechanisms in cellular senescence

Stressors causing unrepairable damage can lead to either cell death or senescence. Although we lack a complete understating on how the decision between those two programs is achieved, entrance into senescence is associated to stimulation of various prosurvival mechanisms that provide enhanced resistance to cell death inducers [70]. Initially, it was shown that senescent cells fail to downregulate the antiapoptotic protein BCL-2 as a consequence of chronic activation of the transcription factor cAMP response element-binding protein (CREB) [71]. More recently, other members of the antiapoptotic BCL-2 proteins family, namely BCL-XL and BCL-W [72], and other key nodes of survival networks, including ephrins, PI3K, p21, and plasminogen activator inhibitor-2 [73] were also suggested to be important players for the resistance to cell death observed in senescent cells. The interaction between FOXO4 and p53 also seems to be an important mechanism in some senescent cells, whereas FOXO4 sequesters p53 in the nucleus preventing its proapoptotic role [74]. HSP90 is upregulated during senescence to maintain the AKT prosurvival signaling pathway activated [75]. P21 can also play a role in this context and protect cells from persistent DNA damage by restricting JNK and caspase signaling [76]. Finally, recent studies demonstrated that the survival of senescent cells is favored by the proper function of Na +/H + ATPase [77, 78].

The discovery of the mentioned survival molecular pathways in senescence established the basics and contributed to the discovery of various classes of drugs that are preferentially toxic to senescent cells – the so-called senolytics.

Dynamics and heterogeneity of the senescence phenotype

Despite major efforts, a unique biomarker that can identify all senescent cells remains elusive [10]. In addition, senescent cells were shown to have multiple functional roles in different situations (Fig. 1). This can also be explained by the heterogeneity of the senescence phenotype which has been now described at different levels.

The variability of the secretory phenotype might be of great interest since it represents a major way by which senescent cells influence their surroundings. Although some SASP factors are common between many types of senescent cells, the general composition of SASP is variable. It is becoming more clear that different senescent cell types develop unique secretory profiles, as measured at both the transcriptional and protein levels [79, 80]. Different inducers of senescence, even in the same cell type, also influence SASP levels and composition. An interesting example of this variable is the difference in SASP production between genotoxic- and mitochondrial dysfunction-induced senescence or "MiDAS," with the latter lacking the NF-κB-driven factors IL-1α, IL-1β, and IL-6, but expressing higher levels of IL-10 and TNFα [81]. More detailed proteomic analysis also revealed differences at the secretion level between commonly used senescence inducers such as genotoxic stress and oncogenic activation inducers [80]. Secondary or paracrine senescent cells also seem to have, in some conditions, a different secretome from the primary senescent cells that led to their induction. Single-cell RNA sequencing revealed that the SASP of secondary senescent cells, mainly induced through juxtacrine signaling, has a noticeable increase in NOTCH-driven genes in addition to a

marked increase in collagen production [82]. Variation within the same cell types exposed to the same inducer of senescence also seems to occur. In fact, the senescence phenotype is now believed to be a dynamic process that can evolve and change with time. For instance, the early senescence state is characterized by upregulation of NOTCH signaling which is positively correlated with the expression of TGF-β and negatively regulates the expression typical inflammatory SASP; thus, NOTCH signaling can act as a switch between two distinct phases of SASP [67]. In contrast, in late senescence LINE-1 retrotransposable elements become derepressed and activate a type-I interferon (IFN-I) response that contributes to the maintenance of the SASP [83]. The unexplored heterogeneity of different secretomes produced by different senescence states can potentially lead to distinct functions in tissue- and context-specific manners that merit further investigation.

Expression of p21 and p16, the most common CDK inhibitors involved in the senescence-associated cell cycle arrest, can also be dynamically regulated in senescent cells. p16 is usually characterized by a late induction, which occurs after an early p53-p21 response responsible for the initial activation of the cell cycle arrest [84]. Additionally, the dependency on one of these CDK inhibitors for the cell cycle arrest is variable among different cell types and even within the same senescent population, where senescent cells uniquely expressing p16 or p21 coexist [85, 86].

As mentioned earlier, although senescence is usually accompanied and induced by DNA damage, some senescence programs do not present signs of genomic damage [21]. Interestingly, this type of senescence seems to have a different functional phenotype (e.g., animal development), which add more complexity to our view of the concept of senescence and suggest the existence of a programmed senescence which induction relies on DNA damage-independent mechanisms.

Another layer of heterogeneity is observed in various prosurvival mechanisms engaged by senescent cells. This is reflected by the fact that senolytics can be broad spectrum but are not able to universally eliminate any type of senescent cell. For example, the survival of some senescent cells depends on the activity of antiapoptotic BCL-2 proteins [87]. For that reason, Navitoclax (ABT-263) was shown be a very effective senolytic in some cell types (e.g., IMR-90 cells) but not in others (e.g., preadipocytes) [87]. Other types of senescence exhibit dependency on another set of survival networks like the PI3K/AKT pathway and dependence receptor/Src kinase/tyrosine kinase [73]. This led to the discovery of new senolytics, like dasatinib and quercetin, that can target senescent cells insensitive to previously identified senolytic molecules. These studies are suggesting that the current available senolytic drugs target senescence in a cell type-dependent manner [87, 88], while it remains to be studied how other intrinsic and extrinsic factors at the core of the senescence heterogeneity influences the response to senolytics.

Based on the fact that many studies are demonstrating the variable nature of senescence, it is logical to suggest that senescence might be a cell fate that includes a collection of heterogeneous phenotypes that reflect the origin and type of cells, nature of the inducer, time after induction, and the interaction with the microenvironment. Therefore, studying senescence from this perspective can help to better understand the complexity of senescence-associated phenotypes and their functional roles, and ease the way for the development of more efficient, more specific, and less toxic senotherapeutics.

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CHAPTER

17

Characteristics of senescent cells

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The time since the last edition of this book has been marked by an exciting series of discoveries in the field of cellular senescence. We have learned that the role of cellular senescence in a living organism is context dependent and the consequences can be very heterogeneous. It has been shown that cellular senescence is instrumental for development, can help regeneration and organ repair, but that it can also be detrimental acting as a major promoter of a variety of rare and common diseases. Other chapters of this book describe in detail the mechanisms and pathophysiological implications of stress-induced cellular senescence in the kidney. Here, we will provide a more general description of the different types of cellular senescence, the phenotype of senescent cells, and the associated functional consequences. On this basis, we will summarize the current knowledge about the impact of cellular senescence in transplantation medicine and we will evaluate perspectives of therapeutic targeting of senescence as a novel strategy to improve transplant outcome.

Definition of senescence

Cellular senescence is a multifaceted process which was first reported in human fibroblasts by Hayflick and Moorhead in 1961 [1]. While culturing multiple strains of human fibroblasts *in vitro*, they observed that the cells failed to multiply after a certain number of passages. When comparing embryonic fibroblasts and fibroblasts derived from adults, they found that embryonic cells were less affected by this phenomenon, which led the authors to call this cell behavior "cellular senescence." A similar *in vitro* pattern has later been confirmed by others in many different cell types, showing that primary cells stop to divide after approximately 50 divisions [2]. Today, 60 years later, the failure to multiply still remains the most important characteristic of a senescent cell. In contrast to the reversible cell cycle arrest of quiescence, which is induced by nutrient starvation and/or the lack of mitogens or growth factors, cellular senescence is permanent and essentially irreversible. Quiescence, in particular in stem cells, is commonly believed to protect cells from going into senescence by reducing the induction of molecular damage (stopping DNA replication, decrease in metabolic activity, reduced gene transcription) [3]. However, interesting recent findings indicate that there might be more overlap between quiescence and senescence than previously thought, suggesting a state of "deep" and long-term quiescence as a transition phase to senescence [4].

In 1965, Hayflick postulated that cellular senescence also occurs *in vivo*, proposing a contribution to organismal aging and exhaustion of tissue repair capacity [2]. At that time researchers faced a problem, which is still a major challenge for senescence research today: How to identify a senescent cell in tissues? Permanent cell cycle arrest as the defining feature is not helpful to this end. Also, the *in vitro* observation that senescent cells spread out on the culture dish and thus increase their cell surface size is not easily transferable to the *in vivo* identification of senescent cells. It was therefore crucial for senescence research that other hallmark features of senescent cells were subsequently found. First of all, it was shown that senescent cells are by no means dormant cells, but that they are transcriptionally and metabolically active. For example, it has been demonstrated in the 1980s that senescent fibroblasts gradually adopt a more glycolytic state [5] and that they alter their secretion of matrix remodeling enzymes, e.g., they produce excessive levels of procollagenase compared to nonsenescent fibroblasts [6]. In parallel, the cells develop increased enzymatic

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activity of the lysosomal hydrolase senescence-associated β-galactosidase (SA-β-GAL), and it was first described by Dimri et al. in 1995 that SA-β-GAL can also serve as a marker for senescent cells on a tissue level [7]. Subsequent comparative microarray studies have demonstrated that the state of senescence is associated with substantially changed transcriptional profiles. For example, Shelton et al. showed in 1999 that senescent fibroblasts express a wide range of proinflammatory genes [8]. This work was later reflected in a seminal work by Coppé et al. in which the researchers extended the previous findings by defining a conserved pattern of secreted molecules which they coined the senescence-associated secretory phenotype (SASP) [9]. Today it is known that the SASP is not always homogeneous but consists of a wide range of secreted proteins which may vary according to affected cell type and context. Typical SASP proteins include cytokines, chemokines, growth factors, and proteases. As the SASP is believed to be crucially involved into the development of age-related disease, it is an important focus of current research and will be discussed in more detail later. Another important finding and a landmark for senescence biomarker discoveries was the observation in 1996 that cyclin-dependent kinase inhibitor p16INK4a is involved in the occurrence and maintenance of cellular senescence [10]. Based on work by Krishnamurthy et al. in 2004 [11], p16INK4a has become one of the most important biomarkers of aging and senescence, and the p16INK4a coding gene locus (CDKN2A) has become an indispensable tool for molecular senescence research over the last years. P16INK4a and other biomarkers of senescence have enabled researchers to prove that senescent cells accumulate with age in many tissues including the kidney [12–14].

Different types of senescence

Developmental senescence

The best known and maybe most logical explanation for an evolutionary benefit of cellular senescence is its protective mechanism against malignancy. Malignant cell transformation and cancer spreading is efficiently suppressed by blocking the proliferative capacity of a potential cancer cell via the senescence pathway (or via apoptosis as an alternative pathway of tumor suppression). However, researchers have discovered other biological and evolutionary beneficial functions of cellular senescence in recent years. In 2013, Munoz-Espin et al. and Storer et al. demonstrated in two seminal papers that cellular senescence occurs widely during mammalian embryonic development [15–17]. Although most of the provided data were from mouse experiments, similar patterns of cellular senescence were seen in chick [16] and human embryos [15], indicating that cellular senescence is a conserved feature of vertebrate development. In elegant experiments, they showed that cellular senescence is part of a developmental program which contributes to remodeling and patterning of the embryo. Of specific interest for kidney researchers, Munoz-Espin et al. studied among other organs the mesonephros, the transitory embryonic kidney which exists before maturation of the permanent metanephros. They found senescent cells in mesonephric tubules by staining for SA-β-GAL and other markers. Importantly, the occurrence of senescent cells was not random, but uniformly and tightly timed just before the developmental regression of the mesonephros begun [15]. They further showed an influx of inflammatory cells and a subsequent clearance of the senescent cells by infiltrating macrophages. Mechanistically they highlighted the functional relevance of p21, showing that developmentally programmed senescence is mediated by p21, while p21 null mice show developmental abnormalities consistent with excess cells. While p21 is also critical in mediating stress-induced senescence (see below), some interesting differences to developmental senescence exist. In contrast to stress-induced senescence, p21 activation in developmental senescence does not depend on the activation of DNA damage response and p53 and also shows no activation of the p16INK4a pathway [18]. Instead, senescence and p21 activation in the embryo seems to be mainly regulated by the TGFβ/SMAD- and FOXO/PI3K-signaling pathways.

While there are little experimental data, yet it is conceivable that inadequate timing, wrong dosing, or spatial dyscoordination of physiologic senescence during development may have detrimental consequences for embryonic programming and for later life [19]. An interesting recent study, for example, found that prenatal exposure to dexamethasone in mice leads to unphysiologically elevated numbers of senescent cells in the metaphysis of long bones, causing bone structural malfunction and postnatal growth retardation [20]. It will be an important topic for future research to investigate whether we can find similar links in kidney disease which might result from alterations in the physiologic programming of developmental senescence.

Replicative senescence

For a long period of time, the term "replicative senescence" was used synonymously with "cellular senescence." It took almost 30 years after Hayflick's first description of "cellular senescence" that Calvin Harley in 1990 provided evidence that the irreversible growth arrest after serial cultivation of mammalian cells resulted from telomere

shortening [21]. Telomeres are protective structures composed of simple repeated DNA sequences at the end of each chromosome. Telomere shortening is a consequence of the end replication problem and occurs in somatic cells without telomerase activity. Only a few years before Harley's description, Carolin Greider and Elizabeth Blackburn had identified the enzyme telomerase and its importance in the elongation of telomeres [22, 23]. For certain primary cells, transfection of telomerase was sufficient to overcome replicative senescence and lead to immortalization [24]. Greider's and Blackburns discovery of telomeres protective nature for chromosomes and the role of telomerase was awarded the Nobel Prize in Physiology or Medicine in 2009 [25]. Meanwhile it has become clear that telomeres are protected by the shelterin complex composed of six proteins (TRF1, TRF2, RAP1, TIN2, TPP1, POT1), which also regulates telomerase [26].

Stress-induced senescence

The concept of senescence has been expanded to include other forms of permanent, irreversible cell cycle arrest which can be reached by DNA damage, oxidative stress, Ras induction, and epigenetic alterations [27–29]. These forms have been synonymously termed as stress or aberrant signaling-induced senescence (STASIS) or stress-induced premature senescence (SIPS). The cell cycle regulator and tumor suppressor p16INK4a is associated with this nonreplication-dependent growth arrest by acting upstream of retinoblastoma [30]. The importance of the INK4a locus in cancer suppression and cell mortality was first described by Serrano and colleagues [31]. Meanwhile p16INK4a has emerged as the marker against which most senolytic therapies are directed [32, 33].

Phenotype of senescent cells and detecting cellular senescence

As of today, there is no unique biomarker which can selectively identify senescent cells. This is particularly challenging for the *in vivo* situation. Most of the classic hallmarks of senescence have been validated in cultured cells *in vitro*, but are not necessarily useful *in vivo*. In cell culture, senescence is accompanied by morphological changes, which are often easy to recognize. Senescent cells become flat, larger, and frequently they have multiple or enlarged nuclei. In most cell types, senescence is also associated with an accumulation of vacuoles and increased lysosomal content [34]. These morphological features which are easy to recognize by microscopic examination allow an initial assessment about the status of cellular senescence. However, for definite identification of the senescent state most researchers rely on a combination of several additional characteristics. (1) The most unified feature is the lack of proliferation markers. Truly senescent cells are unable to enter cell cycle even in the presence of strong mitogens. Therefore, there are no incorporation DNA synthesis markers (e.g., BrdU, EdU) in senescent cells and antibody-based detection of classic cell cycle markers (e.g., Ki67) is negative. (2) Maybe the most widely used senescence assay is cyto- or histochemical staining for enzymatic activity of SA-β-GAL. However, for an adequate use and interpretation of SA-β-GAL as a marker of senescence, it is crucial to pay attention to several points. The staining reaction (using 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside [X-Gal]) for SA-β-GAL per se is not at all specific for senescent cells. In fact, SA-β-GAL activity is found in many normal cells if the staining reaction is performed at a low pH (pH 4.0–4.5), which reflects the normal lysosomal milieu. This "normal" activity is quenched in the SA-β-GAL assay by using a suboptimal pH (pH 6.0). The idea is that under these suboptimal conditions only cells with a very high lysosomal content and hence a very high SA-β-GAL activity still stain positive in the chemical reaction. Thus, SA-β-GAL is a surrogate marker for enhanced lysosomal content and activity. This explains why although SA-β-GAL activity is detected in most senescent settings, it can also be detected in nonsenescent cells. An increased lysosomal content and positive reactivity in the SA-β-GAL assay can, for example, also be found in subtypes of nonsenescent macrophages and osteoclasts [35, 36]. Another disadvantage of SA-β-GAL is that the enzymatic reaction is disturbed by fixatives and cannot be performed in living cells or in paraffin-embedded archival material. (3) γH2AX (phosphorylated H2AX) is a DNA damage marker that can be effectively marked by immunocytochemistry and has been shown to be a promising marker of senescent cells in cell culture and tissues [37]. According to Lawless et al., γH2AX becomes particularly valuable and discriminative for senescence if > 5 nuclear foci can be counted per nucleus [38]. However, nonsenescent cells, such as cancer or proliferating cells, also show γH2AX positivity, and it therefore needs to be confirmed that the γH2AX-positive cell is not proliferating (e.g., by double staining with Ki67). (4) Another important feature of senescent nuclei is the loss of Lamin B1, which is a core structural protein of the lamina enveloping the nucleus [39]. During senescence induction, Lamin B1 expression is downregulated on mRNA and protein level. This change has been demonstrated in different cell types and organs, including the kidney [40]. (5) As mentioned earlier, telomere shortening or telomere damage are

major drivers of replicative and stress-induced senescence. Telomere length can be measured *in situ* (in cells or in tissues) by fluorescent *in situ* hybridization (FISH), which allows detecting age- or stress-dependent accumulations of senescent cells in the kidney [41]. Another way of assessing telomere length is the measurement of telomeric DNA content by quantitative PCR and its expression as a ratio of telomeric DNA to a single-copy gene product [42]. Quantification using PCR has more or less replaced the determination of the terminal restriction fragments using Southern blot [43]. Both approaches use bulk DNA and are therefore not capable of distinguishing certain cell types. (6) Senescence-associated heterochromatin foci (SAHF), which have been linked to the abovementioned nuclear destabilization through the loss of Lamin B1 [44], are facultative heterochromatin domains associated with irreversible cell cycle arrest which accumulate in senescent cells [45]. Structurally, SAHF contain silent heterochromatin domains which can be visualized by foci of intense DAPI staining in combination with specific antibody staining of SAHF components (e.g., macroH2A, H3K9Me2/3, and HP1 proteins) [46]. (7) There is so far no single biomarker protein which is *de novo* expressed in cellular senescence. However, the accumulation of cell cycle inhibitors, which are key regulators of senescent cell fate in the context of DNA damage or other stressors, is a typical feature. Most importantly, the cyclin-dependent kinase (CDK) inhibitors p53, p21, and $p16^{INK4a}$ are frequently used markers of cellular senescence. While antibody-based detection of p53 and $p21^{Cip1}$ is reliable for both human and murine cells and tissues, p16INK4a staining is problematic for mouse tissue and most available antibodies show nonspecific staining. (8) Often it is helpful to include SASP factors into the detection strategy for senescent cells as the expression of many SASP proteins can be readily detected on a cellular level by immunostaining or *in situ* hybridization (however, the SASP "cocktail" may vary strongly according to cell type and kind of senescence induction). (9) A crucial feature of senescent cells, which was first described in 1995, is their resistance to apoptosis [47]. Similar to some cancer cells, senescent cells resist proapoptotic stimuli by upregulating antiapoptotic/prosurvival pathways. In contrast to nonsenescent cells, they keep high expression levels of BCL-2 family proteins, specifically BCL-XL and BCL-W, after treatment with apoptosis-inducing agents [48, 49]. While the detection of BCL-2 family proteins might be an interesting further option to identify senescent cells, there is so far little evidence for the feasibility of this strategy.

Taken together, a definitive and specific marker of cellular senescence has not been identified to date. Researchers have to rely on biological signatures, consisting of the coexistence of several structural, expressional, and functional changes within senescent cells. Given the high variability of senescent cells, which reminds of the large heterogeneity of cancer cells, it is questionable whether it will be possible to find a unifying single senescence marker.

Functional consequences of senescence

Physiology

Cellular senescence is an antagonistically pleiotropic program, comprising benefit versus damage within distinct biological contexts. By blocking cell division in response to genomic, metabolic, proteostatic, or replicative stress, senescence can protect tissues and the organism from transmission of deleterious mutations and propagation of defective cells. Besides apoptosis, which eliminates potential cancer cells by inducing programmed cell death, the permanent withdrawal from the cell cycle by cellular senescence is the second big "gatekeeper" tumor suppressor as summarized by Judith Campisi [50]. Aside from this important tumor suppressor function, senescence has been linked to additional beneficial roles. We have already mentioned the presence and importance of senescent cells during embryogenesis to promote the regression of transient structures (e.g., the mesonephros) and to balance the coordinated development of cell populations [51]. In this context, senescence is p21 dependent and provides through the SASP a mechanism which enables effective cell clearance by macrophages to allow regression and remodeling. Consequently, genetic knockdown of p21 leads to patterning defects in murine limbs [16]. Given the developmental function of senescence in regulating tissue formation, it might be not so surprising that senescence could also play a role in tissue repair and regeneration. However, when Demaria et al. first observed that in the skin senescent cells positively regulate wound healing [52], it was unexpected given the importance of cell proliferation for efficient skin repair. They demonstrated that senescent fibroblasts and endothelial cells appear very early in response to a cutaneous wound and accelerate wound closure by inducing myofibroblast differentiation through the secretion of platelet-derived growth factor AA (PDGF-AA). Thus, PDGF-AA (and potentially other factors of the SASP) dominated the senescence net effect over the antiproliferative break in a subset of cells [52]. Parallel to the developmental observations, it was also shown that transient occurrence of senescent cells can help diminish the overshoot of specific cell populations and thereby prevent excessive fibrosis. This was demonstrated in a liver damage model in which Krizhanovsky et al. found that senescence of stellate cells prevented liver fibrosis and dysfunction by the production of fibrolytic enzymes and by immune cell

attraction and immune-mediated elimination profibrotic cells [53]. While there is no directly comparable data for the kidney, p16INK4a knockout mice have provided heterogeneous results in different renal stress studies. Wolstein et al. found in unilateral ureteral obstruction that p16INK4a knockout mice developed significantly more fibrosis [54]. Using similar mice but a different stress model (ischemic acute kidney injury), Braun et al. observed significantly reduced fibrosis development in association with p16INK4a deletion [55]. Similarly, lack of p16INK4a ameliorated profibrotic changes in a premature senescence model (Bmi-1-deficient mice) [56].

Taken together, beneficial roles of cellular senescence have been described in various conditions, but depending on the time during life span, duration of senescence and level of activation they are in complex balance with detrimental and disease-promoting functions (outlined later).

Pathophysiology

Based on these physiological functions, it is well conceivable that the program of cellular senescence had evolutionary advantages. If senescent cells appear in limited numbers and exist only transiently before being efficiently cleared by the immune system, they are useful in fulfilling important functions in development, cancer control, and potentially in wound repair. With advancing age and in diseased organs, however, senescent cells keep accumulating as a chronic process. Modern techniques, such as single-cell RNA sequencing, have made it possible to study *in vivo* age-related transcriptional changes and changes in senescent load on a cellular level. In the so-called *Tabula muris senis*, it was shown that of all candidates, p16INK4a has the highest correlation to aging and that the proportion of cells expressing CDKN2a/p16INK4a more than doubles over the life span of a mouse [57]. This was accompanied by a twofold increase in the expression levels of p16INK4a in cells in which it was expressed. Interestingly, in very old mice (30-month-old), the fraction of p16INK4a expressing cells slightly declined again compared to 24-month-old mice. It was speculated that this could reflect a cause-effect relationship because long-living animals might have a slower rate of senescence [57]. Despite the abovementioned difficulties of detecting senescent cells *in vivo*, there have also been some important studies in humans which show a similar age dependence. In human kidneys, we have found 20 years ago that telomeres shorten in an age-dependent manner, implying higher frequencies of senescence induction during the aging process [14]. Similarly, expression levels of p16INK4a increased in aged human kidney tissues, showing a strong correlation with typical morphological features of renal aging [12].

What are the consequences of the age-associated accumulation of senescent cells? Experimental data of recent years suggest that the consequences can be correlated to two main mechanisms: (1) intrinsic proliferative deficit; (2) para- and endocrine SASP effects. The first mechanism is linked to the core characteristic of cellular senescence—permanent cell cycle arrest. A cell which is unable to proliferate cannot participate in normal cell turnover of tissue maintenance. Depending on the type of tissue and the respective need for higher or lower cell turnover, this may have organ-specific consequences. In the kidney, where baseline cell turnover is very low, the consequences are thought to be less important during normal organ maintenance but become more relevant during renal repair and regeneration. We have shown that the proliferative defect of tubular cells in telomerase-deficient mice hampers successful repair after ischemic kidney injury, while genetic ablation of CDKN2a/p16INK4a with higher proliferative capacity leads to improved repair [55, 58]. To really understand the functional importance of the cell-intrinsic proliferation deficit, it would be crucial to quantify the actual number of cells affected by senescence within a tissue. To this end, several transgenic mouse lines have been generated using the CDKN2a locus to drive the expression of reporter genes [52, 59, 60]. Liu et al. studied different tissues (not the kidney) comparing young (3 months) to old mice (2 years) and found in the aged group p16INK4a promoter activation in ~ 3%–10% of cells depending on the tissue [60]. In a recent paper, Grosse et al. using another reporter mouse described low numbers of p16INK4a reporter-positive cells in 12 months old mice (< 0.01% positive kidney cells) [61]. Baker et al. used transmission electron microscopy to quantify SA-β-GAL staining on the cellular level of the kidney and reported X-Gal crystals in 1.2% of renal cells in renal cells of 18-month-old mice [59]. In human kidneys, where immunostaining for p16INK4a is more reliable than in mice, Idda et al. recently performed a quantification study showing that cells which express p16INK4a increase with age but are generally rare (up to 0.2% of all cells) [62]. They observed positive p16INK4a staining mainly in tubular cells and sometimes in cells in the Bowman capsule. Even if these quantifications underestimate the occurrence of senescent cells, it seems that very small numbers of cells can have a surprisingly strong impact. This has been impressively highlighted by genetic and drug-based senolytic experiments, in which elimination of senescent cells has extended life and health span and has improved or even reversed the outcome of many age-related disease models in mice (reviewed in Paez-Ribes et al. [63]). In view of these findings, the question arises how very few cells may disturb the function of an entire organ or even systemically impact the whole organism? The current concept assumes that the SASP is the decisive factor.

As the senescent cells persist under pathological or aging conditions, the SASP changes from a transient to a chronic phenomenon. In parallel, the functional consequences shift from beneficial to detrimental. Dysfunctional properties which have been linked to the SASP are manifold and include the secretion of proinflammatory, proapoptotic, procancer, insulin resistance-inducing, immune-cell-attracting, extracellular matrix-destructing, and fibrosis-promoting tumor cytokines, enzymes, bioactive lipids, and miRNAs (reviewed in [64, 65]). Given the continuously expanding list of SASP molecules, it is crucial to stress that the SASP is anything but a fix program. Instead, it seems to vary greatly depending on the type of senescence, the time point during senescence induction, the cell type, the tissue context, and the methodology used in different studies. Hernandez-Segura et al. performed an RNA-sequencing study and meta-analysis of senescent cell transcriptomes and confirmed a great heterogeneity with different patterns of dynamic SASP changes depending on the senescent cell type [66]. In the kidney, there has been little direct research on specific molecules of the SASP. Basisty et al. recently reported a SASP signature of senescent tubular cells [67]. By performing a comprehensive proteomic analysis of soluble proteins and exosomal cargo originating from radiation-induced senescent human tubular cells, they demonstrated the expression of specific new candidates while several prototypical SASP factors were not at all upregulated. While unbiased transcriptome analyses have helped to better understand SASP heterogeneity, future studies will have to directly define and quantify the secreted proteins by proteomic studies. Senescent cells can also secrete exosomes with cargo that has little overlap with classic SASP candidates and might contribute to tumorigenesis and age-associated pathologies [68, 69]. In the context of regenerative nephrology, there are several SASP factors and characteristics which might be of specific importance. In particular, several groups have reported that the SASP has a strong regulatory impact on the cell cycling behavior of surrounding cells. Serpin-B2, for example, is a novel SASP factor [48] which inhibits proliferation in surrounding cells in renal epithelial cultures (own unpublished observation). Importantly, the SASP might even spread the induction of senescence in other cells. Acosta et al. first reported that senescence can be transmitted in a paracrine fashion in several *in vitro* and *in vivo* models of oncogene-induced senescence [70]. They coined this phenomenon paracrine senescence. More recently, their findings have been expanded and it has been shown that the spread of senescence can even occur in an endocrine systemic fashion to induce senescence over distances and tissue borders [70].

An important candidate pathway which is activated by the SASP is WNT/β-catenin signaling. Secretion of several WNT family members has been implicated in the pathogenesis of kidney injury and the aggravation of acute damage. Activation of WNT4/β-catenin signaling correlated with induction of tubular epithelial senescence after ischemic injury [71] and WNT9a/β-catenin activation paralleled senescence induction in several kidney injury models [72]. Overactivation of WNT/β-catenin was also observed in a mouse model with genetic deletion of salvador homolog 1 (SAV1), a component of the Hippo pathway [73]. SAV1 deficiency caused SASP activation that coincided with a dramatic maladaptation and fibrosis after acute kidney injury. Taken together, these data suggest that WNT pathway activation might crucially contribute to senescence-associated aggravation of kidney injury and hamper normal repair.

One of the main characteristics of the SASP which is highly conserved over cell types and types of senescence is the initiation of an inflammatory response. While this proinflammatory secretome is essential for the physiological senescence program (leukocyte attraction and subsequent elimination of senescent cells), the same proinflammatory factors become disruptive in the context of aging and chronic disease. Even small numbers of senescent cells seem to cause a state of inflammation not only in the surrounding microenvironment, but also in the entire organism [65]. Inflammatory cytokines such as IL-6 and IL-8 and others can disturb the process of renal repair by disrupting cell–cell communication, impede macrophage function, and induce innate immune responses [40, 65].

Potential role of senescence in transplantation

Based on the aforementioned, it is obvious that senescent cells may influence different aspects in the transplant setting, in which conditions of the donor as well as the recipient are important predictors of transplant success.

The observation that donor organ quality is a major determinant of graft outcome has been repeatedly shown [74–76] and has led to the definition of so-called extended criteria donors (ECD) [77, 78]. The definition of ECD is closely linked to age and includes either (a) older kidney donors from individuals aged 60 years and above or (b) donors from individuals aged 50–59 years, who also have evidence for two of the following symptoms: hypertension, impaired renal function (i.e., serum creatinine levels above 1.5 mg/dL), or death from cerebrovascular accident [79]. The presence of senescent cells in the kidney with increasing age has been undoubtedly shown not just in animals [11, 13], but also in humans [12, 14]. Hypertension and its vascular sequelae atherosclerosis and arteriosclerosis have been shown to be associated with the occurrence of senescent cells. Arterial hypertension leads to increased p16INK4a expression and fibrotic changes in the kidney, while antihypertensive treatment can prevent these changes

[80]. Shorter leukocyte and intrarenal telomere length has been associated with renal arteriosclerosis at time of implantation [81] and a similar association was seen for intrarenal telomere length in late 5-year transplant biopsies [82]. Senescent cells occur in different compartments of the kidney [12] and their presence has been described for certain kidney diseases [83–85]. Taking all the facts together, it may not be surprising that independent groups were able to show that the amount of senescence in pretransplant biopsies is able to predict transplant outcome [86, 87]. Further support comes from animal studies showing that donor kidneys from $p16^{INK4a}/p19^{ARF}$ KO mice not only result in better survival, but also more proliferative capacity of tubular epithelial cells and less interstitial fibrosis [55]. Recently, the idea that the regenerative limitations of older donor organs through their senescent load can be overcome by treatment with senolytics has been experimentally shown in a cardiac transplant model [88]. Iske and colleagues demonstrated that treatment with dasatinib in combination with quercetin prior to renal IRI significantly reduced systemic levels of cell-free mitochondrial DNA and the expression of $p16^{INK4a}$ in old mice. This reduction went along with reduced systemic levels of proinflammatory T cells.

Older recipient age is also closely associated with transplant outcome and a risk for posttransplant death with functioning graft [89]. It results not only from age-associated changes in the immune response and the interaction with immunosuppression, but also because of increasing comorbidities [90]. It is also likely that immune senescence contributes to certain age-associated phenotypes. DNA methylation age predicted the risk of infection better than chronological age in kidney transplant recipients [91]. In the general population, leukocyte telomere shortening is associated with an increased risk for infection [92] and also for all-cause mortality risk in the general population [93]. The important functional hallmark of immune senescence is refractoriness to antigen signaling and therefore poor response to new immunological stimuli [94]. Underlying changes include thymic involution [95], shifting T-cell populations from lesser naïve T cells toward the accumulation of memory cells against other specificities. In older individuals, B cells display an impaired immunoglobulin class switching that impairs antibody response to the influenza vaccine [96]. However, data from skin allografts indicated that aged B cells were capable of augmenting T-cell alloimmunity in the context of anti-CD20 treatment [97], mainly through a subset of B cells, called age-associated B cells (ABCs), which accumulated during aging [98]. This finding and the observation that transplantation tolerance requires thymic participation and integrated, tolerance-promoting responses to novel antigens [99] is in contrast to the idea that immune senescence in older recipients may protect them through lesser allo responsiveness.

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CHAPTER

18

Stress-induced senescence of tubular cells

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Introduction

Chronic kidney disease (CKD) is common, affecting 850 million people worldwide [1]. It is defined by a persistent loss of renal function and can progress to end-stage kidney disease (ESKD), requiring renal replacement therapy. CKD is thought to occur as a maladaptive response to diverse forms of acute and chronic cell stress, with kidney fibrosis the final common pathway regardless of the underlying etiology [2] [3]. This is characterized histologically by interstitial fibrosis and tubular atrophy (IFTA), and glomerulosclerosis.

The cells of the renal tubule can be subject to a wide variety of stressors. These include, but are not limited to, ischemia, hypoxia, toxins, reactive oxygen species (ROS), inflammatory cytokines, hyperglycemia, and irradiation. A number of intracellular changes can occur as a result. Mitochondrial injury leads to decreased fatty acid oxidation, driving a metabolic switch to glycolysis to meet energy demands. These changes result in increased production of ROS and the NLR family pyrin domain containing 3 (NLRP3)/inflammasomes, leading to the subsequent expression of proinflammatory and profibrotic factors [4]. Renal injury can also result in endoplasmic reticulum (ER) stress in tubular epithelial cells [5, 6] with the accumulation of unfolded and misfolded proteins in the ER. This in turn triggers the unfolded protein response (UPR), which can involve three main signaling pathways involving: protein kinase-like ER kinase (PERK), inositol requiring 1 (IRE1), and activating transcription factor 6 (ATF6) [7]. Persistent DNA damage, which can occur as a result of intrinsic injuries including oxidative damage and telomere attrition or external insults such as irradiation, chemotherapeutic drugs and myoglobin precipitation, triggers the DNA damage response (DDR) [8]. These adaptive responses can help cells recover or lead to apoptosis. An additional cell fate that can occur in response to injury is the induction of cellular senescence [4, 9, 10].

Senescent cells are characterized by irreversible cell cycle arrest, marked changes in their transcriptome and secretome and modifications in their morphology and chromatin organization. Senescence can occur in multiple contexts including serving as a physiological component of healthy organismal development [11, 12]. Whether physiological or pathophysiological, senescence occurs in response to a range of insults including cellular stress, hypoxia, lack of nutrients, genotoxic injury, oncogene activation, and mitochondrial dysfunction [13] (Fig. 18.1). The timing of induction also appears to impact significantly the phenotype and consequences of cellular senescence. In "acute" senescence, the senescent cells appear to have a role in limiting fibrosis in response to injury via fibroblast senescence induction [14, 15] before being removed by leukocytes in a tightly controlled timely manner. Senescent cells can, however, persist and accumulate within affected organs. Unlike other cell fates such as apoptosis, these chronically senescent cells remain metabolically active and through their proinflammatory secretome, known as the senescence associated secretory phenotype (SASP), can act in a paracrine and autocrine fashion to impact adversely the function of tissues and organs (Fig. 18.2).

Senescence is proposed as a potentially important driver of kidney fibrosis, with the epithelial cells of the renal tubule playing a central role [2,16–20]. In this chapter, we review the role of tubular senescence in aging, chronic renal diseases, and in response to acute injury using evidence from studies in humans and animal models.

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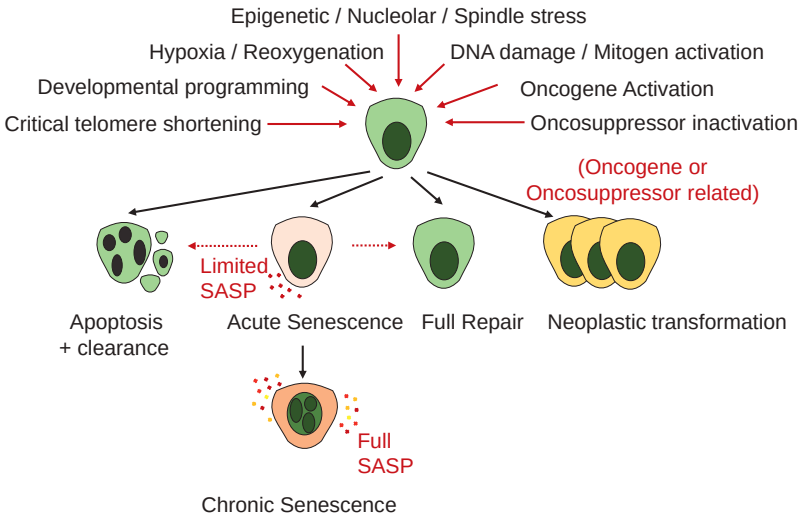

FIG. 18.1 Recognized stimuli to cell fate decisions including apoptosis, senescence, repair, or neoplastic transformation. Multiple developmental, stress-associated or DNA damage-induced cues (red lines) can result in apoptosis, senescence, neoplastic transformation (recognized only in the context of DNA damage–blue line), or full repair. Existing evidence indicates that cells enter "acute" senescence in response to these cues which may lead to repair, clearance, or evolution to the transcriptionally altered "chronic" senescent cell. *Adapted from Docherty M.H., Baird D.P., Hughes J., Ferenbach D.A. Cellular senescence and senotherapies in the kidney; current evidence and future directions. Front Pharmacol 2020;11(755):1–18. https://doi:10.3389/fphar.2020.00755 (https://creativecommons.org/licenses/by/4.0/).*

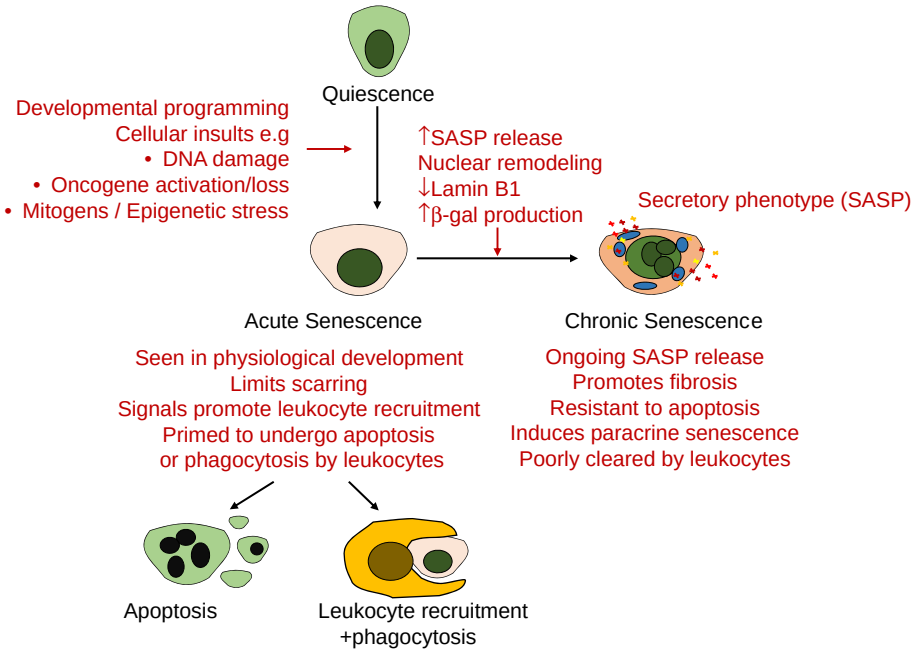

FIG. 18.2 Routes toward clearance or persistence of senescence in vivo. Senescent cells can be generated in response to multiple physiological and pathological stimuli. The canonical pathway involves the onset of acute senescence—a state associated with leukocyte recruitment and clearance of senescence—as seen in development and wound healing in vivo. With advancing age and in response to other stimuli, there is often incomplete clearance of senescent cells, which undergo further phenotypic alterations, upregulate SASP release, nuclear remodeling, and alter the expression of multiple genes including Lamin B1 and β-gal. These chronically senescent cells appear resistant to apoptosis and phagocytosis and are believed to mediate organ dysfunction and fibrosis via their secretory phenotype. Whether these altered outcomes reflect altered initial stimuli, the cell type, the age of the subject, or other unknown factors remain incompletely understood. *Adapted from Docherty M.H., Baird D.P., Hughes J., Ferenbach D.A. Cellular senescence and senotherapies in the kidney; current evidence and future directions. Front Pharmacol 2020;11(755):1–18. https://doi:10.3389/fphar.2020.00755.*

Senescence in human renal disease

The link between aging and senescence is well established in multiple organs including the kidney. Older age correlates with increased senescent marker p16INK4A (measured as both mRNA and protein expression) in the kidney, with the increase particularly marked in the renal tubules [21–23]. Ki-67, a marker of cell proliferation, is inversely correlated with p16INK4A [22].

Renal insults, including hypoxia, subsequent reoxygenation, immune activation and metabolic stress, can induce senescence. There is now increasing evidence that senescent cells, identified by senescence-associated beta-galactosidase (SA-β-Gal), p16INK4A and p21Cip1 expression, accumulate in a number of renal diseases in humans. In each case, senescence is associated with worsened kidney function and outcome. SA-β-Gal reflects the enhanced lysosomal content of senescent cells. p16INK4A and p21Cip1 are cyclin-dependent kinase inhibitors, which induce cell cycle arrest at the G1/S cell cycle checkpoint (Table 18.1). The tubular epithelium is implicated as the primary location of renal senescence [24–30]. Figure 18.3 demonstrates an increasing proportion of p21Cip1-positive epithelial cells as kidney disease progresses in native human kidney biopsies.

The INK4a locus, which encodes for p16INK4A, also encodes for $p14^{ARF}$ in humans and $p19^{ARF}$ in mice, which are structurally related tumor suppressor proteins [31] that may also be detected by antibodies to p16INK4A. While $p14^{ARF}$ in humans and $p19^{ARF}$ in mice are also markers of senescence, this cross-staining raises the possibility of misidentifying the pathways underlying senescence.

In kidney transplant allografts biopsied for abnormal function and found to have tubular atrophy and interstitial fibrosis present, there was a significant increase in nuclear and cytoplasmic p16INK4A staining in tubular cells, when compared to biopsies taken at the time of implantation. The number of these cells was significantly greater than that expected for kidney age [32]. Expression of p16INK4A is significantly elevated in tubular cells (and in glomerular and interstitial cells) in chronic allograft rejection [23] and tubular cells SA-β-Gal expression significantly correlates with the severity of chronic allograft nephropathy [33]. Notably, the presence of SA-β-Gal-positive senescent cells correlated with the age of the donor, but not that of the recipient [33]. Significant telomere shortening has also been observed in patients with delayed graft function, acute rejection, and chronic allograft nephropathy [34]. Furthermore, expression of the gene CDKN2A, cyclin-dependent kinase 2A (which codes for p16INK4A), in

TABLE 18.1 Tissue markers associated with senescence in the kidney.

Marker	Function	Expression in senescence
P16INK4A	Cyclin-dependent kinase inhibitor 2A–blocks cell cycle at G1/S checkpoint	Increased
P19ARF	Cyclin-dependent kinase inhibitor 2A (alternate reading frame)–blocks cell cycle at G1/S checkpoint	Increased
P21CIP1	Cyclin-dependent kinase inhibitor 1A–blocks cell cycle at G1/S checkpoint	Increased
P27KIP1	Cyclin-dependent kinase inhibitor 1B–blocks cell cycle at G1/S checkpoint	Increased
KI67	Nuclear protein associated with cellular proliferation	Absent
SA-β-gal	Lysosomal beta-galactosidase	Increased
LMNB1	Lamin-B1. Nuclear Lamina protein.	Decreased
γ-H2AX	Component of DNA strand break repair machinery	Increased
SAHF	Senescence associated heterochromatin foci	Present

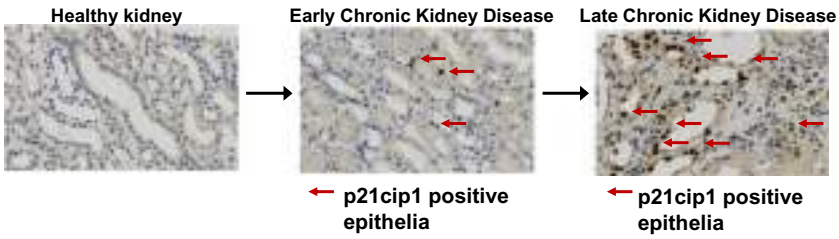

FIG. 18.3 Senescent cells accumulate in progressive chronic kidney disease. Immunohistochemical staining for expression of the cyclin-dependent kinase inhibitor P21CIP1 in native human kidney biopsies obtained at the Royal Infirmary of Edinburgh, Scotland illustrates increasing proportions of P21CIP1 + positive epithelial cells with advancing disease (*red* arrows). *Copied from Docherty M.H., Baird D.P., Hughes J., Ferenb ach D.A. Cellular senescence and senotherapies in the kidney; current evidence and future directions. Front Pharmacol 2020;11(755):1–18. https://doi:10.3389/fphar.2020.00755.*

preimplantation biopsies together with donor age, can predict transplant function at 1 year post transplant in multiple regression analysis [35, 36].

Expression of SA-β-Gal, p16INK4A, and p21Cip1 is significantly increased in human biopsy specimens of patients with IgA nephropathy compared to controls with the renal tubular cells predominantly affected. In addition, the expression of these senescence markers correlated with disease progression and with the amount of collagen III and fibronectin in the interstitium, but notably, not with age, suggesting that the accelerated senescence observed in these patients is explained by the disease and senescence associated with normal aging [37]. In keeping with these findings, patients with IgA nephropathy have shorter telomeres compared to controls [38].

In patients with diabetic nephropathy secondary to type II diabetes, SA-β-Gal and p16INK4A were significantly increased compared to controls. While increased staining for both markers was evident in podocytes, it was particularly marked in the tubular compartment, where SA-β-Gal was increased three-fold in the diabetic nephropathy kidneys. Tubular p16INK4A levels correlated with HbA1c, a measure of diabetic control [39]. The same authors conducted a parallel set of experiments in vitro using primary cultures of proximal tubular cells; they found that passaging cells in high glucose media led to a twofold increase in SA-β-Gal and p16INK4A [39]. This suggests that hyperglycemia may be playing a direct role in the accelerated tubular senescence seen in the patient with diabetic nephropathy. Following on from this, Tsai et al. [40] compared the transcriptome of proximal tubular epithelial cells from a patient with diabetes to a normal individual and through systematic bioinformatic analysis, identified miR-378i and its downstream target S-phase kinase protein 2 (Skp2), as potentially playing a role in hyperglycemia-induced senescence. Notably, in urine from both an animal model of type II diabetes (db/db mice) and human participants with type II diabetes, miR-378i levels were elevated and correlated with urinary senescence-associated secretory phenotype cytokines (including IL-6, IL-8, and MCP-1) and albuminuria. Skp2 levels were decreased in the proximal tubule of both the humans and mice with diabetes. This raises the possibility that urinary biomarkers of senescence might be able to predict disease progression.

In kidney biopsy samples consistent with hypertension and nephrosclerosis, p16INK4A was increased compared to age-matched normotensive controls. Again, levels were highest in the tubules, followed by the glomeruli and then the interstitium [41].

In patients with glomerular disease (focal segmental glomerular sclerosis, minimal change disease, and membranous nephropathy) and tubulointerstitial nephritis, tubular cytoplasmic p16INK4A expression, and glomerular and interstitial cell p16INK4A nuclear staining was increased compared to controls [21]. Differing from other published studies, in this paper, there was no significant increase in tubular p16INK4A-positive nuclear staining, though as noted above, immunohistochemical staining studies may be confounded by cross-reactivity of many antibodies to the related P14ARF protein.

By contrast, in patients with autosomal dominant kidney disease, a genetic condition in which disordered epithelial cell cycle regulation contributes to cyst formation, expression of p21Cip1 in tubular cells was significantly decreased compared to controls [42].

Radiation nephropathy is an established cause of acute and chronic kidney injury [43]. The kidneys are radiosensitive organs. Exposure to irradiation, usually iatrogenic, as for example with total body irradiation prior to bone marrow transplantation, generates oxygen radicals that cause DNA injury within milliseconds. There is often, however, a latent clinically silent phase, which can last a number of years, before the manifestations of kidney disease become apparent, often characterized by extensive kidney fibrosis [44]. Exposure to radiation is known to trigger premature senescence [45, 46] and while a definitive link between radiation nephropathy and senescence in humans has not been shown, evidence from animal models suggests a connection. [47]

Evidence from animal models that senescence predisposes to chronic kidney disease

A key question relates to the true pathogenic role of senescence in the kidney subject to acute or chronic cellular stress. It remains unproved whether, in the clinical setting, the presence of senescent cells is solely a marker of age or previous stress responses, or whether their presence contributes to ongoing cell stress and fibrotic responses. Answering such a question using clinical samples is seldom feasible, and recent studies in animal and in vitro models of cell stress and kidney fibrosis have been instrumental in advancing our knowledge of the importance of stress-induced senescence in the long-term outcome of acute kidney injury.

Several approaches have been used to study renal senescence in animal models. These include animals aged or exposed to external stimuli that trigger accelerated senescence, transgenic mice with changes in their senescence induction pathways, transgenic mice with alterations that facilitate the depletion of senescent cells and pharmacological treatment of mice with agents that target senescent cells and/or their secretome.

The association between age and increased tubular senescence has been demonstrated in several species including mice [48–50], rats [51–53] and cats [54]. Notably, aged mice (46–49 weeks) subjected to ischemia/reperfusion injury had a significantly worse outcome than young (8–10 week old) mice. They had higher levels of p21Cip1, p53, and SA-β-Gal which were associated with more fibrosis, higher creatinine levels, and increased mortality [48]. Echoing human experience, in murine transplantation, allografts transplanted from older mice have higher baseline expression of p16INK4a than kidneys from young mice. P16INK4a increases further by day 7 post transplantation in these older donor kidney, an increase that is not observed in young kidney allografts, suggesting that the older kidneys with higher baseline levels of senescence are also more susceptible to enhanced senescence induction as a result of transplantation [49].

In rats treated with cisplatin to induce tubular damage, p21Cip1 was one of a number of cyclin-dependent kinase inhibitors upregulated in the tubular cells [55]. In keeping with the observational studies in humans, tubular p16INK4a, p21Cip1, and SA-β-Gal expressions were also increased in a rat model of chronic rejection [56].

Irradiation has been used to induce renal senescence in both rat [47] and mouse models [57]. In the latter study, radiation resulted in a decrease in the aging expressed protein, Klotho, as well as an increase in TNFα, a negative regulator of Klotho. Thus, TNFα, in addition to the DDR, may underlie radiation-induced senescence.

A number of animal studies have explored the role of hyperglycemia-induced senescence in the study of diabetic kidney disease [40, 58, 59]. In rats treated with streptozotocin to induce diabetes, increased SA-β-Gal activity was observed in cortical tubules as early as day 10 after treatment [58]. In streptozotocin-treated mice, there was a significant increase in the SA-β-Gal staining of tubular cells 4 weeks after treatment that was associated with an increased expression of p21Cip1. These changes were attenuated by the administration of insulin [59], supporting the concept that hyperglycemia-induced stress served as a pathologically important mechanism in the diabetic kidney.

Several studies have used mice with transgenically modified senescence induction pathways, predominantly p16INK4a and p21Cip1 knockout models, to investigate the role of senescence in renal repair after injury with results mixed, some showing benefit and some harm (Table 18.2) [60–66]. Following unilateral urethral obstruction (UUO), p16INK4a knockout mice had increased tubular and interstitial cell proliferation, reduced apoptosis, and absent SA-β-Gal compared to WT mice. There was, however, greater collagen and fibronectin deposition in the p16INK4a

TABLE 18.2 Transgenic murine models with altered senescence induction pathways and their kidney outcomes.

Reference	Renal model	Modulation of senescence	Renal disease outcome	Effect of intervention or genetic modification
Wolstein et al. [61]	UUO	WT vs P16INK4a KO mice with impaired cell cycle arrest	UUO induces ß-gal positivity, apoptosis and collagen deposition in WT mice	↓ ß-Gal positivity ↓ Apoptosis ↑ Collagen ↑ Proliferation after UUO in P16INK4a KO
Megyesi et al. [62]	Renal IRI	WT vs P21CIP1 KO mice with impaired cell cycle arrest	WT mice show tubular injury and raised blood urea levels after IRI	↑ Proliferation ↓ Renal function ↑ Mortality in P21CIP1 KO
Megyesi et al. [63]	Renal UUO and IRI	WT vs P21CIP1 KO vs P21CIP1 KO + KAP2-driven P21CIP1	WT mice show renal fibrosis after both UUO and IRI	UUO: ↓ Fibrosis in P21CIP1 KO vs WT, ↑ Fibrosis in P21CIP1 KO kidneys with KAP2 promotor-driven P21CIP1 vs P21CIP1 KO. IRI: ↑ TGFß1 in P21CIP1 KO kidneys with KAP2 promotor driven P21CIP1 vs P21CIP1 KO
Lee et al. [64]	Renal IRI	WT vs P16INK4a /p19ARF double KO mice with impaired cell cycle arrest	WT mice show marked P16INK4a and p19ARF induction 28d after IRI, with apotosis and reduced tubular density	P16INK4a and p19ARF deficient mice show improved epithelial and microvascular repair, with increased myeloid cell recruitment
Al-Douahji et al. [65] Wolf et al. [66]	Diabetic nephropathy	WT vs P21CIP1 KO WT vs p27KIP1 KO	WT mice develop albuminuria and glomerular hypertrophy	Both p27KIP1 KO and P21CIP1KO mice were protected from proteinuria and glomerular expansion
Braun et al. [60]	Kidney transplant	P16INK4a KO mice with impaired cell cycle arrest	WT mice develop interstitial fibrosis and tubular atrophy	P16INK4a KO mice develop less atrophy and fibrosis after Tx

knockout mice [61]. Conversely, in mice subjected to ischemia/reperfusion injury (IRI), p16INK4a knockout mice had increased proliferation, reduced IFTA, and improved functional recovery compared to WT mice [60] [64]. Furthermore, mice that received a transplant from p16INK4a knockout mice had a significantly improved survival than mice that received a kidney from wild-type (WT) controls [60]. The increased number of proliferating tubular cells after IRI in the p16INK4a knockout model implies that p16INK4a impairs renal repair after injury by reducing the proliferative capacity of tubular epithelial cells. The increased fibrosis after UUO in p16INK4a knockout mice, however, suggests that different injury cues can cause senescent cells to respond differently, which subsequently impacts the role of these cells in tissue repair and fibrosis. It should be noted, however, that UUO is a very strong profibrotic model and other factors may dominate, preventing antifibrotic effects of a reduction in the senescent response from being manifest.

In p21Cip1 knockout mice subjected to IRI, renal function was poorer and survival lower compared to WT mice [62]. In contrast to p16INK4a knockout mice, however, p21Cip1 knockout mice were protected from renal fibrosis following UUO [63]. Notably, when p21Cip1 was restricted solely to the proximal tubule epithelium of the p21Cip1 knockout mice, there was an increased fibrotic response, which mirrored the WT mice response, demonstrating the key role of proximal tubular senescence in maladaptive profibrotic kidney repair [63], and the apparently discordant outcomes of deficiency in either of the two key pathways of senescence induction. In studies of streptozotocin-induced diabetic nephropathy using both p21CIP1 and P27KIP1 knockout mice, the knockout mice were protected from glomerular hypertrophy and proteinuria. [65, 66]

An alternative experimental approach to inhibiting senescence is to deplete cells after they have become senescent. This approach was first used by Baker et al. [19] with their seminal INK-ATTAC transgenic mouse, whereby the INK4A promoter (a promoter which is transcriptionally active in a population of senescent cells) is used to drive the expression of a transmembrane FK506-binding protein, coupled with intracellular caspase 8. This fusion protein is activated (via dimerization) by dietary administration of AP20817 (AP) and triggers subsequent apoptosis. In AP-treated mice, there was a 75% reduction in senescent cell numbers in the kidney, measured using transmission electron microscopy to count x-gal-positive crystals in renal cells. This was associated with less glomerulosclerosis and lower blood urea levels. Interestingly, even though there was an improved glomerular appearance, the SA-β-Gal-positive cells were found in the proximal tubules, rather than the glomeruli, in keeping with the central role of tubular cell senescence in driving renal fibrosis and the primary role of tubular injury in driving secondary glomerulosclerosis [67]. A similar approach was used by Baar et al. [17] using the P16::3MR mouse model, where the promoter of p16INK4a drives the expression of a trimodality reporter (3MR) consisting of Renilla luciferase (RLUC) for bioluminescence detection, RFP for fluorescence detection, and TK-HSV which can selectively eliminate cells by using ganciclovir. Ganciclovir treatment caused apoptosis in p16INK4a-positive cells in mice treated with doxorubicin. Mice that underwent senescent cell depletion had improved serum urea and creatinine levels.

While informative, it is important to interpret animal models using either global knockout or transgenic depletion with the knowledge that neither p16INK4a nor p21Cip1 is expressed in all senescent cells. Additionally, they may also be expressed in some nonsenescent cells.

Omori et al. used single-cell transcriptomic level analysis to characterize $p16^{high}$ cells in the liver and kidney of p16-CreERT2-tdTomato mice [68]. They found that although $p16^{high}$ cells were present in various cell types, in the kidney they were dominant in the proximal and distal tubules, where there was notable heterogeneity between different $p16^{high}$ cells. The $Tomato^{+}$ proximal tubular cells were enriched for rapamycin-related genes (Atp6ap2, Arf1, Lamtor1, Lamtor4, Nme1, and Nme2) indicating mTOR activation and tunicamycin-related genes (Canx, Ctsd, and Hspa5) indicating a high level of ER stress. The $Tomato^{+}$ distal tubular cells were deficient in cell metabolism and mobility-related genes. In the liver, $p16^{high}$ cells were particularly prevalent in the liver sinusoidal endothelial cells and a subpopulation of Kupffler cells. Eliminating $p16^{high}$ cells ameliorated nonalcoholic steatohepatitis (NASH)-induced liver steatosis and inflammation in a model of NASH [68].

Another method of depleting senescent cells is to target them with pharmacological agents. This approach also has greater translatability as therapeutic interventions in humans. Senescent cells typically upregulate anti-apoptotic pathways and resist apoptosis. Baar et al. [17] utilized one such pathway via p53 to deplete senescent cells. They identified FOXO4 protein as playing a key role, hypothesizing that by binding to p53, this molecule prevented p53 mediated apoptosis. They developed a peptide called FOXO4-DRI, which interferes with the endogenous FOXO4 binding with p53, causing apoptosis. In both fast aging and naturally aged mice, they found a significant increase in SA-β-Gal-positive cells in the tubular cells. They also found a reduction in LMNB1 and an increase in IL-6. FOXO4-DRI reversed these changes; in addition, it led to a decrease in serum urea and creatinine levels seen with aging.

Another senolytic agent, ABT-263 (Navitoclax), promotes apoptosis by targeting the Bcl-2 family (Bcl-2, Bcl-w, and Bcl-xL), depletes senescence renal epithelial cells in vitro [69], but its impact on renal function and fibrosis in vivo is not yet known.

Quercetin, a naturally occurring flavonoid that inhibits phosphoinositide-kinase (PI3K), has also been shown to deplete senescent cells. It has been used to treat mice with accelerated senescence resulting from exposure to 6 months of a high-fat diet [70]. These obese mice have reduced renal function and upregulated senescence markers, including p16INK4a, p19, and p53, in their tubular cells when compared to lean mice. Quercetin reduced several markers of senescence including SA-β-Gal and p16INK4a and led to an improvement in creatinine levels and microalbuminuria that was comparable to the lean controls. Quercetin-treated mice had reduced interstitial fibrosis compared to vehicle-treated obese mice as quantified using Massońs trichrome stain, but there was no difference in collagen-1 expression using immunofluorescence. Investigators in another study using obese mice combined quercetin with dasatinib, a tyrosine kinase inhibitor which blocks Ephrin B-dependent suppression of apoptosis, found that this regimen leads to an improvement in microalbuminuria [71]. The investigators also noted a reduction in p16INK4a-positive cells in the renal cortex and an increased expression of Wilms tumor protein (WT-1), a measure of podocyte function.

As a cautionary note, however, in a recent study by Grosse et al., using a transgenic mouse model targeting p16INK4a [72], the authors found that the majority of p16INK4a expressing cells were vascular endothelial cells (and to a lesser extent, macrophages and adipocytes). Their removal was associated with worse outcomes, including liver fibrosis, though kidney outcomes were not reported. Another study by Demaria et al. suggested an important role for senescent cells in skin repair. Using the p16::3MR mouse model, elimination of senescent cells delayed wound healing [73].

Tubular senescence in Acute Kidney Injury

The kidney, unlike the heart and brain, has the capacity to recover from injury. Tubular epithelial cell proliferation, which normally occurs at a low baseline rate [74], dramatically increases following ischemic or toxic injuries, to replace the cells lost to necrosis and apoptosis [75]. Genetic fate mapping studies in transgenic mice subjected to ischemic injury have confirmed that the source of these replenishing epithelial cells are mature cells that have survived the insult, which de-differentiate prior to proliferating to replace the lost cells [76]. It is, however, increasingly recognized that in humans the kidney often does not fully recover following an insult but rather, that acute kidney injury (AKI) leads to the acceleration of CKD [77, 78].

CKD progresses to end-stage kidney disease often due to processes unrelated to the original disease with tubulointerstitial fibrosis the predominant underlying pathology. The decline in renal function, however, is often nonlinear with AKI being a major risk factor for developing end-stage kidney disease. This is particularly pronounced in patients who already have underlying CKD when they develop AKI [79] and in the elderly [80], who are also more susceptible to developing AKI [81, 82].

Maladaptive repair following tubular injury, resulting in the development of fibrosis, underlies this relationship between AKI and CKD [2,20]. It was previously thought that epithelial to mesenchymal transdifferentiation (EMT) was one of the major pathways in fibrosis [83]; however, multiple studies indicate that myofibroblasts, the cells responsible for collagen deposition, do not derive from proximal tubule cells in lineage-tracing experiments [76] and are generated instead from multipotent stromal progenitor cells (pericytes) [84, 85] in response to signaling from the tubular epithelium [20, 86]. A fundamental question in the pathogenesis of renal fibrosis following injury, which remains incompletely understood, is what molecular mechanisms determine whether the tubular cells pursue a reparative response or profibrotic process ensues. Renal senescence, induced by injury, may play key a role in determining the outcome (Fig. 18.4). CKD can be considered accelerating aging of the kidney [2].

Purported features of renal tubular epithelial senescence

As we have described above, senescent cells accumulate in the aged and injured kidney. The SASP, which is initiated in part through NF-κB [87], includes a wide range of proinflammatory cytokines, chemokines, growth factors, and proteases, which act in an autocrine and paracrine fashion to mediate many of the effects of senescent cells [88] [89]. While SASP signaling can promote tubular repair after injury in the short term, it also promotes a proinflammatory and profibrotic microenvironment in the longer term [16].

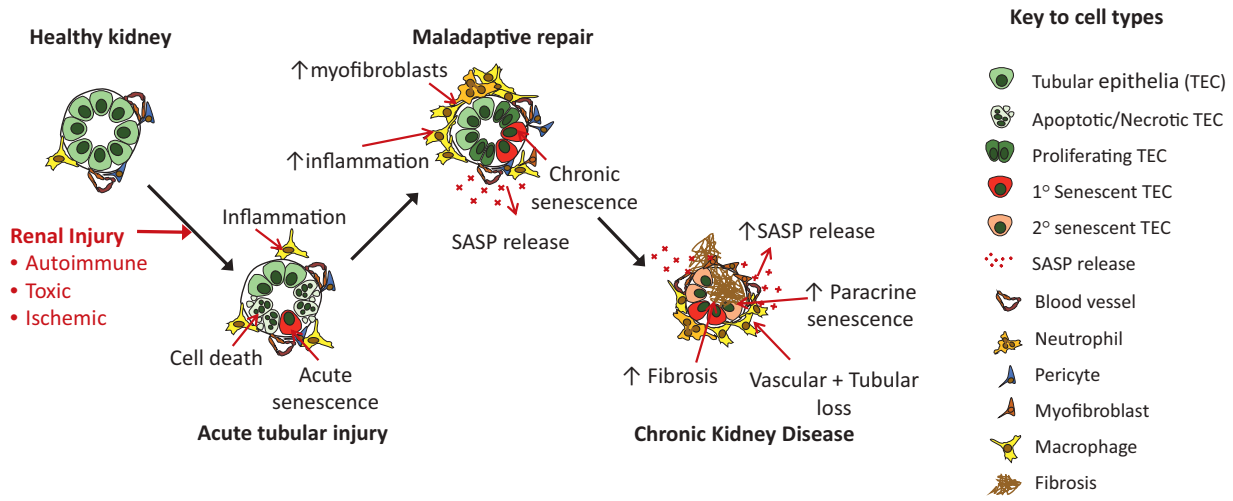

FIG. 18.4 Putative roles for senescent cells in the evolution of maladaptive repair after acute kidney injury. Our understanding of the role of senescence in the evolution of maladaptive repair after injury remains incomplete. Evidence from clinical studies identifies increasing numbers of senescent cells in the aged and chronically damaged kidney, and experimental models show reduced renal fibrosis after senolytic treatments. In this diagram, we outline potential mechanisms by which senescent cells generated in response to aging or injury may promote ongoing fibrosis via SASP release leading to immune activation, fibroblast proliferation, and further induction of paracrine senescence. *Adapted from Docherty M.H., Baird D.P., Hughes J., Ferenbach D.A. Cellular senescence and senotherapies in the kidney; current evidence and future directions. Front Pharmacol 2020;11(755):1–18. https://doi:10.3389/fphar.2020.00755.*

Senescent tubular epithelial cells appear within a few days of kidney injury. In one experimental study, the authors found that this response was mediated by epithelial Toll-like and interleukin 1 receptors (TLR/IL-1R) of the innate immune system [90]. Inhibition of epithelial cell innate immune signaling in mice by knockout of myeloid differentiation 88 (*Myd88*) prevented the accumulation of senescent tubular epithelial cells, reduced damage to kidney tubules, and ameliorated fibrosis following folic acid-induced injury. Notably, however, when the inactivation of *Myd88* was triggered 5 days after injury, there was an equally effective reduction in senescent epithelial cells and fibrosis, but no protection from tubular damage at 28 days after injury, suggesting that early intervention after injury is required [90]. In this study, the authors also report that senescent cells are a source of profibrotic Hedgehog ligands, sonic (Shh) and Indian (Ihh) hedgehog.

Tubular epithelial Notch1 signaling has also been linked with senescence and a profibrotic phenotype. There is prolonged Notch signaling activity in the kidneys of old mice following ischemic injury. Induced expression of the Notch intracellular domain in proximal tubules was associated with enhanced tubular injury, a fibrotic phenotype and increased levels of p21 and p16INK4a. Inhibition of Notch in WT mice reduced expression of p16INK4a and p21Cip1 and reduced tubule-interstitial damage [91]. The authors conclude that sustained Notch signaling after IRI may lead to the prosenescent phenotype and maladaptive repair.

Wnt9a expression in human tubular cells is increased in a number of renal diseases and correlates with p16INK4A expression and the degree of fibrosis. Through a combination of experiments in vitro using human epithelial cells and in vivo in mice, Luo et al. demonstrated that Wnt9a upregulates the senescence phenotype in tubular cells including production of TGF- β1, while shRNA-mediated knockdown of Wnt9a repressed renal fibrosis following IRI [92].

G2/M cell cycle arrest in response to sustained renal injury

While classically senescence-inducing stimuli result in cell cycle arrest at the G1/M checkpoint (as measured using p16INK4a and p21Cip1), there is also accumulating evidence that suggests cells arrested later at the G2/M checkpoint play an important role in the development of fibrosis following injury [20, 93–96]. In multiple models of AKI (ischemic, toxic and obstructive), G2/M arrested cells upregulate profibrotic cytokine production including TGF-β1 and CTGF via JNK signaling leading to increased fibrosis. Reversing G2/M arrest, using JNK or p53 inhibitors, protected the kidney from fibrosis following injury [20]. Epithelial cells in the G2/M phase form target of rapamycin

(TOR)-autophagy spatial coupling compartments (TASCC), with cyclin G1 (CG1) playing a key role. Deletion of CG1 prevented G2/M arrest, inhibited TASCC formation, and ameliorated renal fibrosis after injury [97].

Biomarkers of tubular senescence

At present, the established markers of senescence rely on tissue for immunohistochemical staining or gene expression using quantitative polymerase chain reaction. In humans, this requires a renal biopsy, which is usually only performed in a subset of patients due to the risk of major bleeding complications. Tubular epithelial cells are in direct contact with formative urine, so it may be possible that analysis of the urinary secretome will allow the level of senescence in the kidney to be quantified noninvasively.

Urinary (and plasma) levels of p21cip1 are significantly increased following AKI [50]. Moreover, in vitro studies of the supernatant of senescent renal epithelial cells suggest the SASP is much larger and more diverse than originally thought, with over 300 proteins either significantly up- or downregulated following senescence induction via irradiation [98]. Notably, the SASP was different in fibroblasts and varied depending on the senescence inducer. As these proteins are secreted by senescent tubular epithelial cells, they may be detectable in urine and allow not only for senescence to be quantified but provide more temporal information about senescence and propensity to fibrosis within the kidney allowing for noninvasive monitoring.

Conclusions

Tubular senescence is now well documented to occur in the aging kidney, in acute kidney injury associated with IRI or toxins and in chronic renal diseases, as well as after kidney transplantation. Senescent epithelial cells play a key role in mediating fibrosis with animal models helping us to better understand the molecular mechanisms driving this process. Senescent cells represent a novel therapeutic target to minimize maladaptive profibrotic repair and promote adaptive repair after injury and hence prevent fibrotic kidney disease progression.

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CHAPTER

19

Stress-induced senescence as a forme fruste of chronic kidney disease—A case for failed regeneration

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Introduction

Chronic kidney disease (CKD) afflicts more than 13% population globally [1]. The list of conditions culminating in CKD includes primary and secondary glomerulopathies, tubulointerstitial, nephrotoxic, systemic diseases, and acute-to-chronic kidney disease continuum. Remarkably, CKD is accompanied by multiorgan involvement—heart, vasculature, accelerated atherosclerosis, endocrine, musculoskeletal, neural, adipose. These comorbidities are so pervasive that it has been proposed to introduce the term cardio-kidney-damage (C-K-D) [2]. What could be the cause(s) of multiplicity of affected organs in kidney injury? Among possible contenders, the cell senescence and accompanying it secretion of senescence-associated secretory phenotype products that affect all cells, including stem cells, and leading to accelerated organ aging is gaining appreciation. In such a scenario, senescent or stress-induced prematurely senescent kidney-resident cells by secreting an array of proinflammatory, profibrogenic products, such as interleukins 1, 6, and 8, connective tissue growth factor, transforming growth factors, plasminogen activator inhibitor 1, MCP-1, GM-CSF, G-CSF, HGF, IGF, PDGF, VEGF, and MMPs, collectively designated "senescence-associated secretory phenotype" products (SASP), induce autocrine, juxtacrine, and paracrine signaling to affect a broad range of local and distant cells. Here, I shall present data to support the idea that many cases of CKD are, in fact, forme fruste of cell senescence or stress-induced premature senescene and therapeutic implications of this conceptual shift.

Multiple points of convergence between CKD and cell senescence

The features of CKD significantly overlapping with senescence or stress-induced premature cell senescence (SIPS) underlie the proposed view of CKD as a forme fruste of premature senescence or premature kidney aging.

CKD is broadly defined [3] as "kidney damage for at least 3 months, … increased number of systemic complications (cardiovascular disease [CVD], hypertension, mineral and bone disorders, and anemia), morbidity and mortality associated with declining eGFR, and … a greater risk of death from CVD than from progression to kidney failure and ESRD." Notably, the prevalence of senescent cells in the renal parenchyma, predominantly in the cortex, is increased in all compartments: glomerular, tubular, and vascular [4–6].

Cell senescence, i.e., aging kidney, on the other hand, is characterized as a mild loss of function in the absence of identifiable underlying illness [7]. The functional attributes of aging kidney are represented by the increased proportion of senescent cells in the kidney, declining GFR and higher prevalence of microalbuminuria or albuminuria [8]. Morphologic changes in aging kidneys and CKD kidneys are also remarkably similar: glomerulosclerosis, tubular atrophy, interstitial fibrosis, and microvascular rarefaction [7]. Chronological aging remarkably resembles SIPS, in

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that the markers are similar, both impinge upon kidney functions, and have generalized, in many instances deleterious, effects via senescence-associated secretory products.

Several physician-scientists have pointed out to a significant similarity between aging-associated decline in kidney function and cell senescence [4, 9–11]. Here, I would like to carry this similarity further to echo Wang et al. [12] and suggest that chronic kidney disease is an imprint of inciting causes of renal disease richly impregnated with senescent cells or, metaphorically speaking, "a palimpsest with senescent cells written all over it" [13].

Endostatin and tissue transglutaminase in aging kidney: A paradigm of kidney senescence with broader implications

The list of geronic proteins is growing to include insulin-like growth factor-1, Klotho, and many others, but here I would like to focus on endostatin and tissue transglutaminase (TG2) as paradigmatic of the role played by geronic proteins in cell and organ senescence. Lin et al. [14], while examining aging mice, have noted upregulation of collagen XVIII and its product endostatin. Endostatin, the natural angiogenic inhibitor, is a 20–22 kDa C-terminal fragment of collagen XVIII [15], a ubiquitous component of subendothelial and subepithelial basement membranes, especially in vessels with fenestrated endothelium, like those in endocrine glands, liver sinusoidal cells, renal glomeruli, and peritubular capillaries [16]. Cleavage of collagen XVIII is accomplished by the action of cysteine proteinase cathepsin L, matrix metalloproteinases (-3, -7, -9, -13, -14, and -20), and the serine protease elastase [17, 18]. Tumor suppressor p53 induces synthesis of collagen XVIII and proteolytic cleavage of its C-terminal domain—endostatin [19, 20]. Endostatin functions as a powerful inhibitor of endothelial cell proliferation, blocker of endothelial and endothelial progenitor cells functions (migration, survival, vessel stabilization, and angiogenesis), and inducer of endothelial cell apoptosis. Endostatin binds to receptors 1–3 for vascular endothelial growth factor and interferes with their signaling [21, 22]; interacts with integrins α5β1 and αVβ3 leading to inactivation of RhoA and disassembly of F-actin and focal adhesions [23]; it inhibits cyclin-D1 promoter and causes G1 arrest of cell cycle in endothelial cells [24], and promotes endothelial-to-mesenchymal transition [25]. Endostatin may have diagnostic value since its urinary excretion is elevated in patients with chronic allograft dysfunction [26].

We compared circulating levels of endostatin in young and aging mice of two different strains and showed that endostatin levels are elevated in the latter [14]. Renal expression of endostatin increased approximately 5.6-fold in aging animals. This was associated with microvascular rarefaction and progressive tubulointerstitial fibrosis. In parallel, the levels of sirtuins 1 and 3 were significantly suppressed in aging mice in conjunction with the expression of markers of senescence. Young mice receiving endostatin for 28 days showed delayed recovery of circulation after femoral artery ligation and reduced patency of renal microvasculature, but no significant tubulointerstitial fibrosis and microvascular rarefaction in the kidney of endostatin-peptide treated young mice. Notably, the patency of and the blood flow in the peritubular microvasculature was impaired. We have conceptualized these findings in aging mice to represent a case for antagonistic pleiotropy, i.e., the dormancy of scattered malignant cells being achieved by suppressing angiogenic cues and curtailing their blood supply, but the achieved angiogenic dormancy due to the intrinsic production of angiogenesis inhibitors, like endostatin, affects and compromises *pari passu* the renal microcirculation [27].

However, aging is not the only stimulus for endostatin overproduction. Endostatin is elevated, both locally and systemically, in the course of diverse pathologic processes. Increased local production of collagen XVIII and endostatin has been documented in conditions as varied as liver cirrhosis, ischemic cerebral events, and hypobaric hypoxia, among others [28–30].

Could endostatin excess be involved in chronic kidney disease? Is there a role reversal for endostatin in young animals to become a precursor of chronic kidney disease? Moreover, is elevated endostatin sufficient and necessary for inducing kidney disease? We have shown that 28-day continuous endostatin delivery via an osmotic minipump reduces renal microvascular patency, but does not cause fibrosis and renal dysfunction [14]. However, when a nephrotoxic stressor was superimposed, endostatin-receiving mice showed a more profound injury. Transgenic mice overexpressing endostatin showed a modest renal interstitial fibrosis already at the young age [14].

One of the recently described molecular partners of endostatin is tissue transglutaminase (TG2) [31], a multifunctional protein richly represented on the surface of vascular endothelium and in the extracellular matrix, where it interacts with fibronectin, collagen, osteopontin, heparin, and syndecan-4 [32, 33]. The main function of TG2 on the cell surface and in the interstitium is catalysis of Ca^{2+} dependent cross-linking reaction of extracellular matrix proteins to stabilize them by forming covalent γ-glutamyl-N^{ε}-lysine bond between acyl donor and acyl acceptor and scaffolding for cell adhesion [34]. TG2 has been shown to participate in fibrotic processes: inhibition of TG2 reduces tubulointerstitial fibrosis and preserves function in experimental chronic kidney disease models [35–37]. It is also a subject of

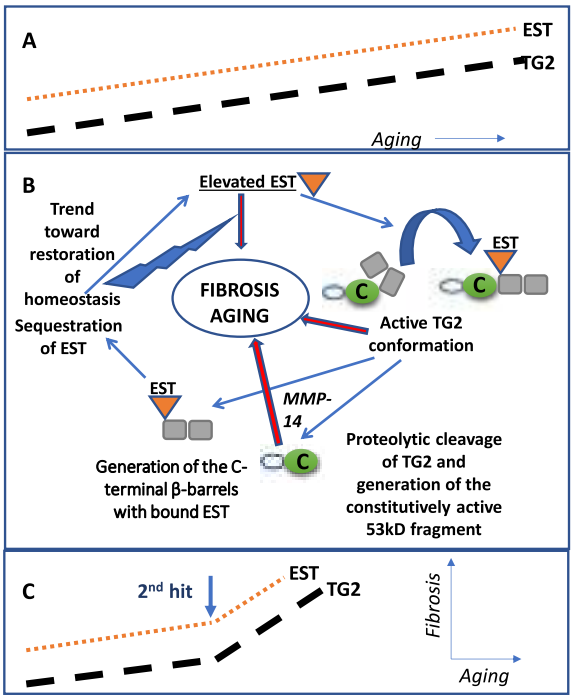

FIG. 19.1 Dynamics of and interactions between endostatin and tissue transglutaminase in aging. (A) Elevation of endostatin and tissue transglutaminase levels in the kidney with aging. (B) Interactions between endostatin and tissue transglutaminase. Tissue transglutaminase is schematically represented as catalytic domain C and two β-barrels. Endostatin binds to the C-terminal B-barrel domains of tissue transglutaminase and keeps it in an open (active) conformation. The possible consequences of endostatin binding: keeping transglutaminase in the active conformation; making transglutaminase more susceptible to cleavage to produce the constitutively active 53 kDa fragment; sequestering soluble B-barrel domains from inhibiting tissue transglutaminase (53 kDa) activity. (C) Noxious stimuli (the second hit) accelerate the rise in endostatin and tissue transglutaminase, thus leading to fibrosis and stress-induced premature aging. Abbreviations: *EST*, endostatin; *MMP-14*, matrix metalloproteinase-14; *TG2*, tissue transglutaminase.

proteolytic degradation by MMP-14 leading to enzyme activation [38]. We have previously demonstrated, as summarized in Fig. 19.1, (1) significantly increased levels of TG2 and its constitutively active 53 kDa fragment in aging murine kidney; (2) endostatin alone contributes to a modest interstitial fibrosis; (3) exaggerated fibrotic response accompanies folic acid-associated nephropathy on the background of elevated endostatin level in mice, especially during the early, "wound healing" phase of nephropathy; and (4) excess of endostatin or TG2 induce premature senescence or apoptotic death of kidney-resident cells [39]. These studies allowed us to conclude that kidney fibrosis in aging may represent a natural outcome of upregulated endostatin and TG2, but, much more likely, it appears to be a result of cumulative renal stresses occurring on the background of elevated and synergistically acting geronic proteins, endostatin and TG2. Hence, while the contribution of geronic proteins is sufficient to induce a mild fibrotic response, the unequivocal development of fibrosis leading to organ dysfunction would require a self-amplifying and perpetuating action of an additional noxious stimulus to lift the senescent cell burden above a subclinical threshold.

Such a scenario involving geronic proteins as a background for the promotion of kidney fibrosis upon encounter with nephrotoxic stressors may have a broad applicability even in young animals. In particular, it may illustrate the Janus-like nature of some forms of chronic kidney disease to metamorphose between forme fruste and forme plaine, as was elegantly construed for focal and segmental glomerulosclerosis by Yee [40]. Namely, a forme fruste may convert into forme plaine by incurring a nephrotoxic stressor and, upon elimination of the stressor, may undergo the reversal to forme fruste. Understanding the mechanisms behind such a reversal and its occasional failure may have therapeutic implications.

The fate of senescent cells—From rejuvenation to persistence of senescent state or cell death

The repertoire of renal-resident senescent cells is schematically illustrated in Fig. 19.2. The fates are not invariable and transitions among them may occur. For the sake of clarity, some interconversions are omitted, such as, for instance, the possibility of an irreversibly senescent cell to eventually undergo apoptosis. An intricate interplay

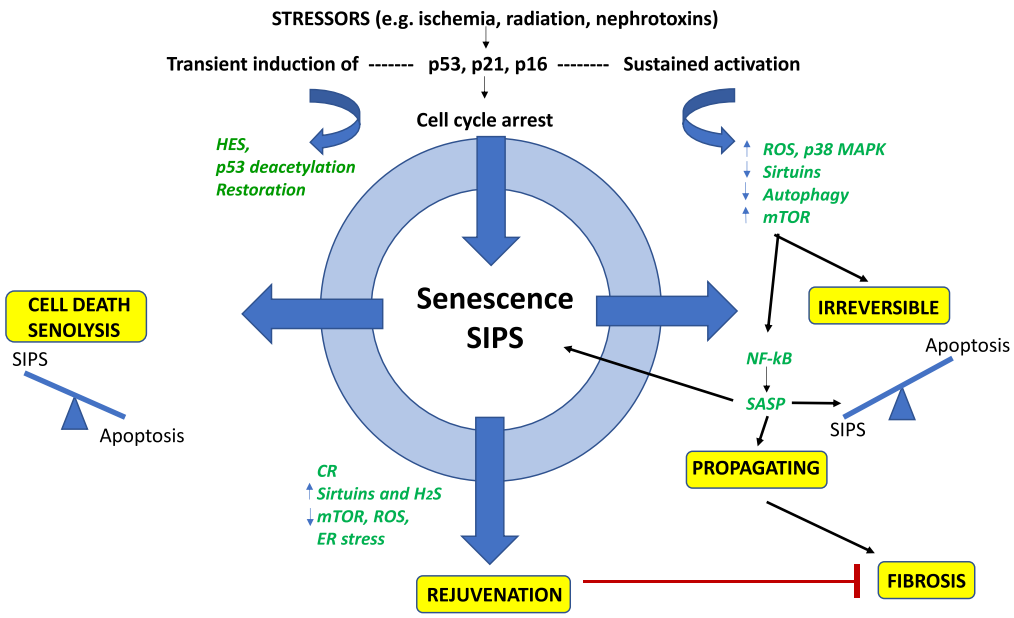

FIG. 19.2 Molecular mechanisms leading from noxious stressors to cell senescence or premature cell senescence (SIPS), their reversibility and persistence. (Individual mechanisms are shown in *green*.) Potential sequelae of cell senescence and SIPS, such as irreversibility, propagation of senescence, cell death and senolysis, and rejuvenation are shown in *yellow boxes*, as is the outcome of protracted senescence—fibrosis.

between cell stressors, transient as opposed to sustained signaling to cell cycle regulators, pro- and antiapoptotic stimuli, and metabolic aberrations are endowed with regulating the trajectories of cell fate (Fig. 19.2). Induction of cyclin-dependent kinase (CDK) inhibitors p53, $p21^{CIP1}$, and $p16^{INK4a}$ leads to cell cycle arrest. Upregulation of these inhibitors occurs as a component of DNA damage response, as in the radiation-induced premature senescence involved in glomerular diseases [41]; telomere attrition as a result of excessive cell division; and cell stressors, like oxidative stress, accompanying SIPS. The role of reactive oxygen species is illustrated by the finding that fibroblasts obtained from highly regenerative mammals, like spiny mice and rabbits, are resistant to oxidative stress, rapidly detoxify H_2O_2 as compared to wild-type counterparts, and show lesser increases in p53, $p21^{CIP1}$, p19, and $p16^{INK4a}$ and development of cell senescence [42]. Pretreatment of wild-type fibroblasts with *N*-acetyl-cysteine afforded similar protection. This observation transcends connective tissue fibroblasts as *N*-acetyl-cysteine is also protective against renal interstitial fibrosis in cisplatin-induced renal senescence [43]. Induction of CDK inhibitors represents a default adaptive response to the stressors, which halts division of damaged cells and destines them to apoptotic cell death or to premature senescence. Decreased c-Myc expression promotes oxidative stress-induced SIPS via a telomere-independent mechanism mediated by a member of polycomb group histone methyltransferase, Bmi-1, and a member of gate-keeping tumor suppressor, $p16^{INK4a}$, in human fibroblasts and endothelial cells [44]. Downregulation of Bmi-1 by a ring finger protein Mel-18, linked to downregulation of c-Myc, results in accelerated senescence [45]. In stress situations, increase in p38 MAPK, ataxia-telangiectasia mutated kinases activity, and a burst in reactive oxygen species result in enhanced phosphorylation of p53, $p21^{CIP1}$, and $p16^{INK4a}$, thus delaying their degradation, inhibiting CDK and causing hypophosphorylation of retinoblastoma tumor suppressor Rb and inhibition of cell cycle progression (reviewed in Ref. [46]).

The very same stress factors are capable of inducing either proapoptotic signals (phorbol-12-myristate-13-acetate-induced protein, Bcl-2 homology domain 3 only protein, and p53-upregulated modulator of apoptosis) or antiapoptotic signals (B-cell lymphoma 2 family members). The balance between them determines whether cells undergo apoptosis or acquire resistance to it. In the case of resistance to apoptosis, combined with the cell cycle arrest, premature cell senescence ensues. A biphasic hormetic effect of p53 activation has been described: while sustained induction of p53 predominantly leads to apoptosis or senescence, lower and transient increases predispose to only a temporary cell cycle arrest [10, 46, 47]. Acetylation of p53 has been identified as a driver for premature renal cell senescence in cisplatin-induced nephropathy; in turn, the protein deacetylase sirtuin 1 activation, followed by p53 deacetylation, results in reduced cell senescence and fibrosis [48]. These findings emphasize the role of sirtuin-dependent posttranslational modification and point out to deficiency in sirtuins as a contributor to irreversibility of senescence phenotype in such conditions. Senescence-promoting pathways, like $p16^{INK4a}$, ARF-p53,

or FOXO-reactive oxygen species, are repressed by the activity of Polycomb group proteins, as argued by Sharpless and DePinho [49]. These regulators are linked to the Forkhead box transcription factor FoxM1c, and its deficiency results in SIPS. Elevated levels of $p16^{INK4a}$ constitute a hallmark of senescent cells, in both replicative and premature senescence. In fact, genetically triggered clearance of $p16^{INK4a}$-expressing cells delays the onset of aging-associated disorders [50].

The Delta-like 1-induced senescence [51], in contrast to the $p16^{INK4a}$-dependent cell-autonomous mechanism of senescence, represents an example of the noncell-autonomous mechanism. In this noncell-autonomous category, senescent cells reinforce and propagate signals, such as inflammatory cytokines, TGF-β, Dikkopff-1 antagonist of Wnt signaling, insulin, and insulin-like growth factor-1 to coerce increasing numbers of neighboring cells to cell cycle arrest and functional demise of the organ [50]. Activation of mTOR pathway serves as a modulator of Wnt/β-catenin pathway [52]. In addition, it regulates SASP by promoting interleukin (IL)-1A translation [53]. In turn, mTOR is activated by insulin and insulin-like growth factor-1 (IGF-1) signaling pathways which contribute to premature senescence. Targets of mTOR include inhibition of autophagy, regulation of ribosomal biogenesis, protein translation, mitochondrial metabolism, and lipid synthesis. The mTOR regulation of autophagy is accomplished via shuttling on and off the lysosomal membrane: in its active state, mTOR is associated with lysosomes and mediates anabolic effects, whereas its dissociation from the lysosomal membrane signals catabolic states.

Autophagy plays either an adaptive role supplying cells with nutrients or it may be conducive of apoptotic cell death in acute and chronic kidney disease [54, 55]. Defective autophagy is responsible for the accumulation of unfolded proteins in the endoplasmic reticulum and induction of unfolded protein response. Antiapoptotic Bcl-2 proteins, often elevated in senescent cells, interact with the autophagy protein Beclin1 and inhibit formation of autophagosomes, thus further disabling autophagy. In a mutant mouse strain deficient in Klotho, a model of premature senescence, Wnt pathway is chronically activated. Alternatively spliced Klotho gene results in the synthesis of a membrane form of the protein (a coreceptor for FGF23) and a secreted form (regulates activities of insulin and IGF-1). The retinoic acid-inducible gene-1 (RIG-1) has been shown to be suppressed by the intracellular form of Klotho. Moreover, RIG-1 is an inducer IL-6 and IL-8 and consequently, defective Klotho signaling re-activates RIG-1 and enhances secretion of IL-6 and IL-8, both proinflammatory components of the SASP [55].

The role of SIRT1 in cell fate and metabolic regulation is based on its effects augmenting FOXO-regulated stress resistance, interaction with and deacetylation of p53, E2F1, PGC-1a, PPAR-γ, eNOS, and NF-kB, to name a few [56–59], thus regulating cell viability, mitogenesis, proinflammatory and metabolic pathways. Sirtuins deacetylation pathway is linked to the mechanistic target of rapamycin (mTOR) and its effects on autophagy [60]. Inhibition of autophagy has emerged as a powerful proaging mechanism [61]. Calorie restriction by suppressing mTOR prevents aging-associated glomerulosclerosis in rats [62]. The mTOR and sirtuins pathways are rendered interactive via the energy-sensing AMP-activated protein kinase (AMPK), as sirtuin 1 deacetylates liver kinase B1, an activator of AMPK, thus actuating it [59]. The opposite is also true: AMPK modulates mTOR activity via its upstream regulator tuberous sclerosis complex 2 (TSC2) and Raptor component of mTOR complex 1 [63, 64]. This site may represent the nexus where AMPK interacts with the Wnt pathway, potentially involved in the persistence of senescent state after an insult. Given mTOR activation in senescent cells, it is not surprising that they also show enhanced Wnt signaling, and Wnt9a has been linked to induction of premature senescence and fibrogenesis in kidneys subjected to ischemia-reperfusion injury [65]. *Ex juvantibus* inference from the fact that rapamycin and its analogs, rapalogues, prevent chronological aging-associated disorders tends to explain increasing attention to this class of drugs as pharmaceuticals for use in kidney disease, not only in transplantation but also in polycystic kidney disease, renal cell carcinomas, and diabetic nephropathy [66, 67]. Notably, factors responsible for persistence of senescent state and/or SASP are also considered inducers of fibrogenesis [68]. In addition, cell senescence is a cause of defective regeneration [68]. In conclusion, a plethora of interconnected mechanisms divert protractedly stressed cells from reversal of cell cycle arrest and rejuvenation toward premature senescence and accumulating burden of an organ with senescent cells.

Local and systemic effects of senescent cells

One of the questions concerning an indolent course of senescence-associated kidney disease, albeit with an exaggerated response to nephrotoxic stressors, consists in the pathogenic importance of senescent cells. A plausible answer lies in the overall senescent cell burden. In an elegant study by Karin et al. [69], the turnover of senescent cells was found to be slower in aged mice compared to young mice. Turnover slowing down was mainly due to the reduced rate of clearance, leading to a fluctuating accumulation of senescent cells until their numbers reach a threshold. The curves of accumulation of senescent cells recapitulate the Gompertz empirical sigmoidal curve

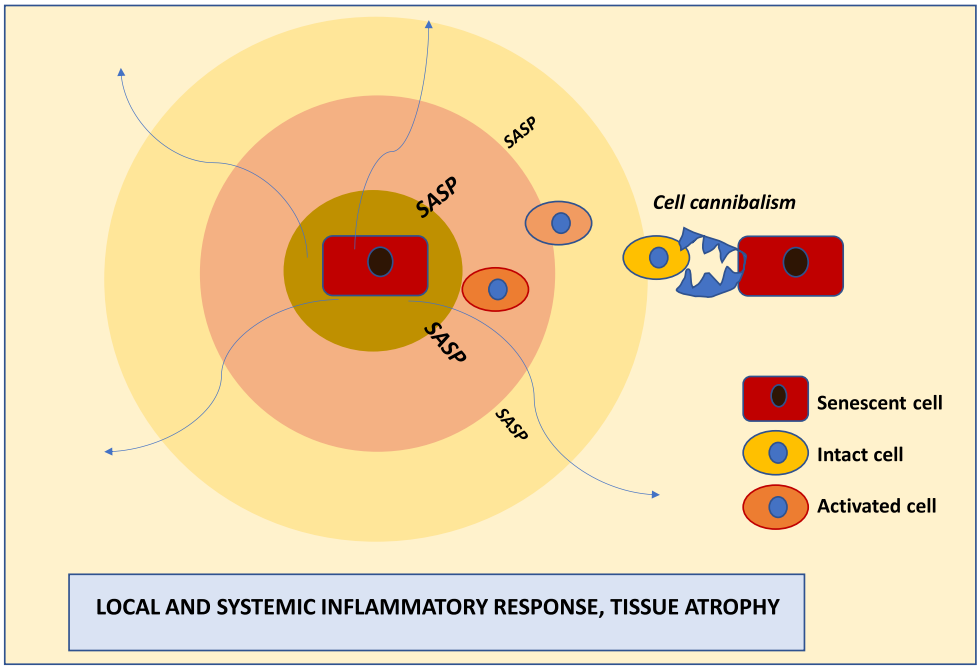

FIG. 19.3 Senescent cell burden. Senescent cells produce senescence-associated secretory phenotype products affecting neighboring cells. Moreover, senescent cells may acquire cannibalistic behavior to engulf and digest neighboring intact cells. Both characteristics, when reaching a threshold, result in local and systemic inflammation and tissue atrophy.

reaching a plateau; this curve describes a law of human mortality [70]. Akin to population dynamics and mortality predictions based on Gompertz curve, the numerical burden with senescent cells could affect organ and ultimately organismal function. In addition to a numerical burden of senescent cells (which by itself limits organ functions), their products also determine their pathogenicity. Cell senescence is characterized by the secretion of SASP proteins [71]. Its common components, which differ depending on the cell type and stressors involved, include profibrotic and proinflammatory agents, such as interleukins 1, 6, and 8, connective tissue growth factor, transforming growth factors, plasminogen activator inhibitor 1, monocyte chemoattractant protein-1, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, hepatocyte growth factor, IGF, platelet-derived growth factor, vascular endothelial growth factor, matrix metalloproteinases, all endowed with paracrine, autocrine, and juxtacrine actions. This composition of SASP underlies the persistence of senescent cells and propagation of its deleterious effects on neighboring cells. In addition to the above components of SASP, senescent and/or stressed cells secrete other products, cumulatively designated as a secretome, which are capable of exerting proinflammatory and profibrotic effects [72–75] by interfering with the Wnt, Notch, and glycocalyx rheostat.

An additional unexpected mode of senescent cell's action has been described [76] in senescent cancer cells capable of engulfing neighboring cells. This behavior does not appear to be limited to cancer senescent cells and may well prove to be common to all senescent cells [77]. Such a cell cannibalism by senescent cells may explain development of atrophy and substitution of parenchyma with connective tissue (as is evident in sarcopenia of aging.) The described sequelae of senescent cell burden (Fig. 19.3) are schematically depicted and should explain the emerging therapeutic strategies. Collectively, the data emphasize the propensity of senescent cells to compound the local and systemic injury.

Therapeutic corollaries

As noted above, transitions between the states of "Forme Plaine and Forme Fruste" in the course of CKD are possible and one of therapeutic goals may represent the right shift of this relation. By analogy with the mortality actuary curve of Gompertz, where shifts in either direction will herald prolongation or shortening of the lifespan, similar shifts applied to the population of senescent cells could translate into changes in organ or organismal function (Fig. 19.4). Intercurrent infections, ischemic episodes or nephrotoxic stressors, obstruction and other noxious stimuli could shift this curve to the left, similar to the "second hit" modeling in the cases of endostatin, or TG2, or SIPS. In

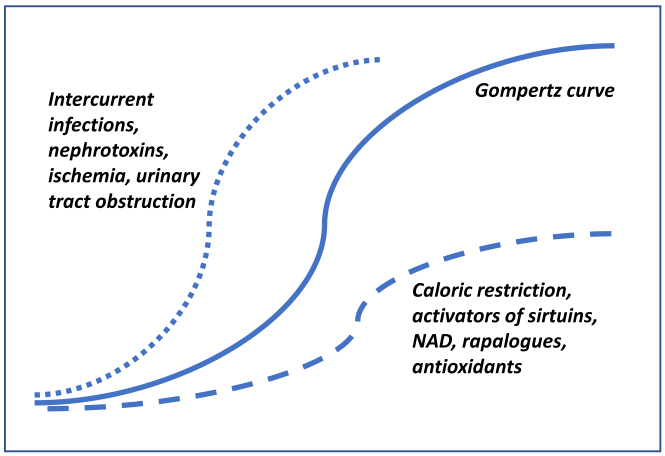
Intercurrent infections, nephrotoxins, ischemia, urinary tract obstruction
Gompertz curve
Caloric restriction, activators of sirtuins, NAD, rapalogues, antioxidants

FIG. 19.4 Application of Gompertz equation to cell senescence. *Left shift of this curve*, representing forme plaine, is induced by noxious stimuli and heralds accelerated senescence. *Right shift*, analogous to transition to forme fruste, may be achieved by judicious administration of several therapeutic strategies.

contrast to this, caloric restriction, rapalogues, sirtuin activators, antioxidants, and other maneuvers are capable of the right shift of Gompertz curve, as they could improve renal function. It is apparent that the causes leading to the left shift tend to accelerate the aging phenotype in the young population, the situation similar to SIPS. While individual therapeutic strategies will be elucidated to a great detail in other chapters (Chapters 6.28–6.32), here they are enumerated to merely support the concept of forced transition toward forme fruste.

Recently developed senotherapies, including rapalogues, are potentially capable of promoting the conversion to forme fruste [4, 10, 78, 79]. Antiinflammaging effects of long-term caloric restriction leading to activation of sirtuins and overexpression of single immunoglobulin IL-1-related receptor and blockade of TLR-4/NF-kB pathway have been recognized and could potentially promote transition to forme fruste [56]. Recent clinical trials of a variant of caloric restriction, alternate day fasting which reduces caloric intake by 37%, showed a remarkable amelioration of physiological and aging markers [80]. Some antioxidants tested in aging mice have been shown to reduce senescence of parietal epithelial cells and attenuate glomerulosclerosis [81]. Activation of sirtuins, an example of rejuvenating strategy that also reduces oxidative stress and ameliorates proinflammatory components of SASP, may serve the same purpose (reviewed in Ref. [82]). NAD^+, a cofactor necessary for activation of several sirtuins, bioavailability of which is reduced in disease states and aging could be supported by supplementation with its precursor, nicotinamide [83]. Repurposing of a biguanide antidiabetic drug metformin to combat aging, as has been established in the large clinical trial TAME (targeting aging by metformin), is based on its beneficial effects on improving nutrient sensing, stimulation of autophagy, prolonging life span of stem cells and improving mitochondrial functions, among others, has been advocated [84].

In conclusion, I presented early evidence to support the concept that some cases of CKD represent a forme fruste of cell/organ senescence, while in aging kidney development of a mild renal dysfunction is similarly explainable on the basis of cumulative effect of senescent cells and their secretomes, like SASP. I have illustrated the poignant issue on the role played by some geronic proteins to cause a modest kidney dysfunction and fibrosis, whereas the florid unveiling of chronic kidney disease requires a second hit which potently converts geronic proteins into profibrogenic messengers. In cases of a preexisting disease, these geronic proteins may be induced already at young age, leading to similar consequences. An array of molecular mechanisms are employed in the persistence of cell senescence. The Janus-like nature of CKD—in that it may present itself as forme fruste or forme plaine—justifies attempts of therapeutic conversion of the latter to the former state. Only future studies could establish the validity of such an approach.

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CHAPTER

20

Premature vascular aging and senescence in chronic kidney disease

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Introduction—CKD and vascular phenotype

Patients with chronic kidney disease (CKD) have a substantially higher cardiovascular risk compared to subjects with preserved renal function [1–3]. According to the Global Burden of Diseases, Injuries, and Risk Factors Study 2017 [3], CKD is the 12th leading cause of death in 2017 and is projected to be one of the five most common causes of death globally 2040. Furthermore, CKD contributes to a reduced quality of life of affected patients both directly through symptoms of advanced CKD or treatment-related effects and indirectly through associated comorbidities [4, 5]. When patients have progressed to end-stage kidney disease (ESKD) and renal replacement therapy (RRT) by dialysis, cardiovascular events and mortality rates are extremely high [6, 7]. However, the pathophysiological mechanisms of the high mortality in CKD and especially ESKD have not been fully understood and, consequently, therapeutical efforts cannot sufficiently reduce mortality risk. For instance, an adverse lipid profile, including total cholesterol [8] and triglycerides [9], predicts increased vascular [8] and overall [9] mortality while lowering of low-density lipoprotein (LDL) cholesterol decreases mortality in the general population [10]. In contrast, hypocholesterolemia was an independent predictor of death in a Japanese study [11], and higher levels of lipid parameters were independently associated with reduced all-cause, cardiovascular, and noncardiovascular mortality in a recent European multicenter study comprising of 5382 incident hemodialysis (HD) patients [12]. These data are further supported by negative outcome trials of statins in HD-patients [13,14] suggesting modified and/or additional components in the development of cardiovascular diseases (CVD) in patients with CKD and ESKD. In this chapter, we summarize the novel concept of vascular senescence and early vascular aging (EVA) as an important risk factor for the development of premature cardiovascular complications and provide an overview on potential novel targets for the systemic treatment of EVA in CKD.

Premature vascular senescence in chronic kidney disease

In the past, cardiovascular risk in patients with or without CKD was commonly attributed to atherosclerosis, i.e., the accumulation of fatty/fibrous material in the tunica intima of arteries [15]. However, arteriosclerosis, i.e., a functional and phenotypical disturbance of the media vessel layer [16], has subsequently been recognized to be a major pathological feature in CKD patients with [17] and without [18] ESKD. Importantly, arterial stiffness as a consequence of arteriosclerosis is strongly related to mortality in ESKD [19]. In addition, atherosclerosis and arteriosclerosis frequently coexist in CKD despite different pathophysiological mechanisms and risk factors [20] further increasing cardiovascular risk in these patients. As age is a major predictor for arteriosclerosis [21], the concept of EVA was proposed and, consequently, distinct disease states were related to EVA [22,23], including age [21], hypertension [21], diabetes [22], and CKD [24,25].

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CKD and associated comorbidities show many similarities with the aging process, for instance a high prevalence of CVD, sarcopenia, mineral and bone disorders, as well as frailty [26,27], cognitive dysfunction [27], immune deficiency [28], and finally mortality [3] in affected patients, suggesting that patients with CKD undergo a process of premature aging. We, therefore, summarize current concepts and pathomechanisms of EVA in CKD, including uremic toxins, senescence, inflammation, klotho deficiency, and repressed expression of nuclear factor-erythroid 2-related factor (NRF2), all linked to hallmarks of the aging process [29]. Translationally, prevention and treatment of EVA are crucial to reduce the burden of disease in CKD, and we highlight potential, holistic approaches for inhibiting EVA.

Early vascular aging in chronic kidney disease

In the uremic milieu, EVA is a concept of premature and adverse vascular aging/senescence that is linked to mortality [19,30]. In general, vascular aging comprises of at least two different phenotypes, i.e., arterial stiffness and endothelial dysfunction (ED) [31]. Arterial stiffness commonly determined by pulse wave velocity (PWV) is pathophysiologically reflected by an extensive vascular calcification (VC) in the tunica media and vascular smooth muscle cells (VSMC), and changes in the extracellular matrix (ECM) are the main drivers on the cellular level [32]. In a healthy individual, VSMC show a contractile phenotype mediating the Windkessel function of large arteries. In contrast, VSMC undergo an adverse phenotypical switch and becomes dysfunctional in CKD [32]. Furthermore, elastin as a major component of the ECM is degraded [33]. ED is a further mechanism involved in EVA that is characterized by a reduced nitric oxide (NO) bioavailability. Consequently, Donato et al. [31] define age-related ED as an impaired vasodilation or a reduced NO bioavailability. ED commonly measured by flow-mediated dilatation also associates with cardiovascular events and mortality in patients with [34] or without [35] CKD. We discuss the effects of several factors on both hallmarks of EVA, i.e., arterial stiffness and ED.

Uremic retention solutes and EVA

With declining renal function, several organic compounds accumulate in the circulation of affected patients [36,37], which can be sorted based on their respective physico-chemical characteristics into the three groups: (1) water-soluble low-molecular-weight solutes (< 500 Da); (2) protein-bound solutes; and (3) middle molecules (500–58,000 Da) [36,37]. The biological impact/toxicity of these uremic retention solutes are comprehensively reviewed elsewhere [37]. For a number of these compounds, associations with EVA and VC have been described [38]. It should be emphasized that not only decreased renal clearance but also increased tissue generation (such as reported for cytokines, FGF23, and adipokines) contribute to systemic levels of uremic toxins.

The water-soluble low-molecular-weight asymmetric (ADMA) and symmetric (SDMA) dimethylarginine both reduce the bioavailability of NO in endothelial cells [39,40]. Consequently, ADMA and SDMA were associated with increased mortality in CKD [41,42]. Trimethylamine-N-oxide (TMAO) has been associated with renal impairment in humans and different animal species [43]. This gut microbiota-dependent metabolite was further shown to induce VC in VSMC through nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome activation, and nuclear factor κB (NF-κB) signaling [44] potentially establishing this small water-soluble uremic toxin as a promoter of both inflammation and EVA. Since TMAO levels in part reflect intake of red meat, it is of interest that a recent study including six prospective US cohort studies reported that higher intake of processed meat, unprocessed red meat, but not fish, was significantly associated with increased risk of incident CVD [45].

It has been reported that high urea levels in CKD induce insulin resistance and increase the risk of incident type 2 diabetes [46]. High concentrations of urea observed in CKD can also induce irreversible carbamylation, i.e., a nonenzymatic posttranslational modification of proteins or amino acids, via isocyanic acid [47]. As an example, carbamylated low-density lipoproteins (LDL) directly cause ED [48], endothelial cellular senescence [49], and significantly predict cardiovascular events and all-cause mortality in CKD [48]. Further data show that protein carbamylation also exacerbated the calcification of human VSMC [50] indicating that carbamylation has deleterious effects of both major components of EVA. Advanced glycation end products (AGEs) are modified sugars and biomolecules by nonenzymatic glycation reactions [51,52] that accumulate with decreasing renal function due to both reduced renal clearance and increased formation [51]. AGEs contribute to both ED and VC (reviewed in Ref. [51]). For the protein-bound uremic toxins, indoxyl sulfate (IS) and *p*-cresyl sulfate (PCS), a large number of studies have described mostly adverse effects in patients with CKD [37]. Both IS and PCS have been shown to induce VC in the medial layer of different arteries in rats with CKD [53,54]. Mechanistically, a proteomic approach suggests activated inflammation and coagulation pathways to be involved in the observed associations [54]. Furthermore, IS induces cellular senescence in VSMC [55] and both IS and PCS also have adverse effects on the endothelium [53,56]. Several

cytokines belonging to a group of middle molecules are also recognized as uremic toxins, including tumor necrosis factor (TNF), interleukins (IL), and adipokines [36,37,57]. Besides their specific inflammatory [57] or metabolic [58] associations, for instance, TNF [59,60] and TNF-like weak inducer of apoptosis (TWEAK) [61] have been directly linked to the components of EVA. For the adipokine chemerin, conflicting data on both components of EVA, i.e., VC and ED, have been reported [62–64].

In conclusion, and despite conflicting results for a few compounds, a significant number of uremic retention solutes/uremic toxins that accumulate with failing kidney function exert direct, adverse effects on EVA [38]. Targeting uremic toxins generally (e.g., by RRT lowering many compounds) or specifically (e.g., by biologicals inhibiting one distinct toxin), has the potential to decrease EVA and, thereby, reduce the high burden of CVD in CKD.

Inflammation, oxidative stress, and early vascular aging

In a large proportion of patients with advanced CKD, a systemic inflammatory response can be detected in the circulation that increases with the progression of CKD [65,66]. Mechanistically, chronic inflammation in CKD is caused by the uremic milieu (e.g., uremic toxins, AGEs), a leaky gut with gut dysbiosis, hypoxemia, exogenous factors (e.g., dialysis treatment, vascular access), secondarily by the underlying CKD-causing diseases and other comorbidities, as well as cellular factors (e.g., oxidative stress) [65,67,68]. Inflammation has been associated with EVA and premature aging in other organs in different human cohorts; a phenomenon denoted "inflammaging" [69]. Regarding CKD, a small cross-sectional study suggests that proinflammatory $CD14^+$ $CD16^+$ monocytes associate with PWV in predialysis patients [70]. Furthermore, systemic IL-6 predicts poor outcome [71] and progression of vascular disease [72] in ESKD patients. Moreover, Desjardins et al. [73] confirm an association of circulating IL-6 with PWV as a marker of EVA. It should be noted however, that in the CRIC study, no longitudinal associations of various inflammatory cytokines with markers of arterial stiffness were found [74]. Further data from a randomized controlled trial with the IL-1 trap rilonacept or placebo for 12 weeks indicate that antiinflammatory treatment improved ED, but not VC [75]. TNF is known to decrease NO bioavailability, thus promoting EVA [76]. Importantly, selective blockade of TNF, but also IL-1, in uremic serum prevents the adverse, osteoblastic phenotype switch of vascular progenitor cells [77]. This is in accordance with findings from the CANTOS trial in patients with CKD where inhibition of IL-1β reduces major adverse cardiovascular event [78]. Angiopoietin 2 (Ang2) is another cytokine potentially linking CKD and inflammation to EVA. Thus, Ang2 is increased in patients with CKD and induces profibrotic changes in aortic VSMC, as well as proinflammatory chemokines in EC, thereby adversely affecting EVA [79]. As medium-sized arteries from children with CKD show increased oxidative DNA damage and senescence markers as features of EVA [80], the uremic milieu has proatherogenic effects also in the absence of traditional Framingham risk factors. Furthermore, in vitro results indicate that VSMC senescence in ESKD induces a proinflammatory phenotype, i.e., senescence-associated secretory phenotype (SASP) [80] suggesting that vascular inflammaging is a bidirectional process. Although acute inflammation is a key biological response to injury that promotes healing, a chronic low-grade inflammatory response is not part of the natural healing process, but instead represents a pathological alteration that correlates with the accumulation of senescent cells. Hyperfunctional senescent cells [81] mediate vascular dysfunction in part via secretion of inflammatory mediators (i.e., SASP), which poison the surrounding tissue and inhibit progenitor cells [82].

Oxidative stress is another prominent feature of CKD that can lead to CKD-related complications characteristic of premature aging, including inflammation, fibrosis, anemia, protein-energy wasting syndrome, and EVA [83,84]. In general, patients with CKD show increased oxidative stress based on an overproduction of reactive oxygen species (ROS), as well as decreased antioxidant defenses [83,85]. Regarding EVA, oxidative stress has long been known as a major risk factor for vascular dysfunction in patients with CKD [86,87]. Oxidative stress can either directly or indirectly (i.e., being a mediator of other CKD-related comorbidities and disturbances) induce EVA by different mechanisms. Direct effects of augmented oxidative stress include a dose-dependent increased VSMC calcification after treatment with hydrogen peroxide (H_2O_2), as well as a phenotypical switch from a contractile to an osteogenic VSMC phenotype as assessed by Runt-related transcription factor 2 (RUNX2) protein expression [88]. In the context of CKD, expression of osteoblast differentiation-related markers was increased in a rat model of CKD and diabetes mellitus [89]. Conversely, treatment with the antioxidant apocynin lowered oxidative stress, osteoblast differentiation-related markers, as well as aortic calcification [89], a finding which was recently validated [90].

Indirectly, oxidative stress can link several CKD-related comorbidities and metabolic disturbances with EVA. For instance, the uremic toxin IS can induce EVA through oxidative stress and indirect stimulation of proinflammatory cytokines. An oral adsorbent of IS improved ED and reduced oxidative stress in patients with early CKD [56]. Results in an uremic mouse model [91], as well as in human umbilical vein endothelial cells (HUVECs) [92], confirm these findings. Furthermore, AGEs also show detrimental effects on ED, i.e., NO bioavailability, which are mediated by increased oxidative stress [93].

The endocrine phosphate-FGF23-klotho axis and early vascular aging

The endocrine phosphate-fibroblast growth factor-23 (FGF23)-klotho pathway crucially influences phosphate metabolism and is dysregulated in CKD [67,94]. Within this axis, phosphate homeostasis is regulated by vitamin D, parathyroid hormone (PTH), klotho, and FGF23 [67]. Dietary inorganic phosphate is absorbed by the small intestine [95] and vitamin D metabolites can increase the rate of intestinal phosphate resorption [96]. Conversely, parathyroid glands sense elevated phosphate levels and release PTH enhancing urinary phosphate excretion [97]. Bone-derived FGF23 further limits hyperphosphatemia by inducing phosphaturia and suppressing vitamin D secretion [98]. FGF23 signals through endocrine FGF receptor complexes [99] and requires klotho protein as a co-receptor [99]. Klotho protein is predominantly expressed in the renal tubuli [100] and circulates as a soluble klotho fragment serving as a surrogate marker of renal klotho expression [99]. All members of this endocrine phosphate-FGF23-klotho axis can influence EVA and are subsequently discussed (Fig. 20.1).

In general, an impaired renal phosphate clearance in CKD produces a relative overload of phosphate, which results in hyperphosphatemia and contributes to inflammation, progression of kidney disease, ED [101] and VC, as well as EVA [94]. Whereas a Scottish general population study links phosphate to markers of "whole-body" biological age (i.e., reduced telomere length, DNA methylation content, and chronological age [102]) data from a Dutch, population-based cohort study [103] demonstrate that higher serum phosphate concentrations, even if they are within the normal range, are also associated with ED. In vitro experiments showed that phosphate treatment arrests the cell cycle mediating cellular senescence in human aortic VSMC [104] (Fig. 20.1). Phosphate further induces VC and oxidative stress and additionally exerts negative effects on EVA through Ca-phosphate crystals and a disturbed microRNA homeostasis [94] (Fig. 20.1). Mechanistically, the adverse effects of phosphate on EVA are at least in part mediated by the Wnt/beta-catenin pathway [105,106]. Thus, high phosphate activates the Wnt/beta-catenin pathway in VSMC in vitro promoting VSMC calcification and the phenotypical switch from a contractile to an osteogenic VSMC phenotype [105]. Interestingly, klotho is not only acting as a co-receptor for FGF-23 but also is an inhibitor of the Wnt/beta-catenin pathway [99] ameliorating high phosphate-induced VSMC calcification [107]. Despite some promising experimental data, two systematic reviews and meta-analyses did not find strong evidence for a beneficial effect of vitamin D supplementation on arterial stiffness [108] and ED [109], i.e., the major components of EVA (Fig. 20.1). The bone-secreted FGF23 is a key protein involved in the endocrine phosphate-FGF23-klotho axis [67] that limits hyperphosphatemia by inducing phosphaturia [98]. Increased FGF23 levels observed in CKD are associated with cardiovascular and noncardiovascular mortality [110], due to noncausal effects [111] or direct effects on the heart promoting left ventricular hypertrophy [112]. Diverging results on FGF23 effects on EVA have been shown. Whereas FGF23 increased VC in a rat CKD model [113], and one study linked the calcification of the vascular tissue with the expression of FGF23 in the vasculature [114], exogenous FGF23 did not induce VC in human VSMC [115].

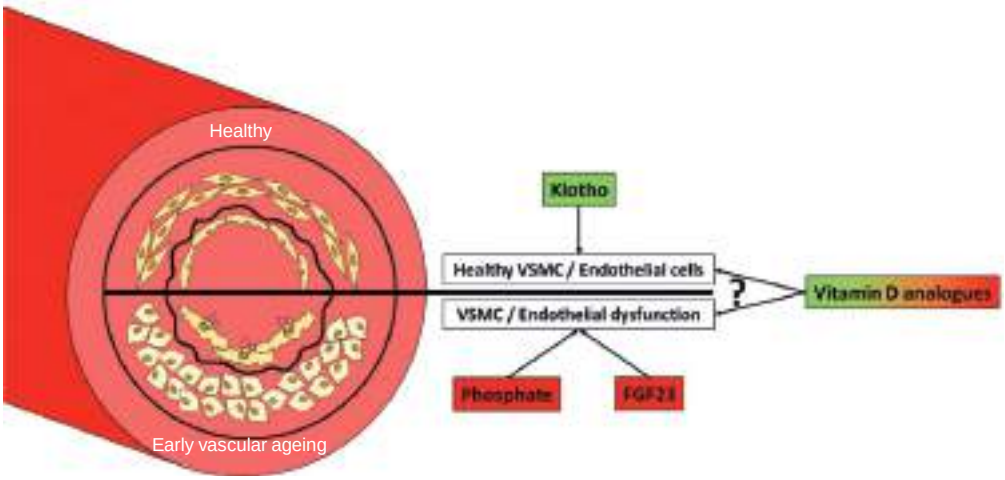

FIG. 20.1 The endocrine phosphate-fibroblast growth factor 23 (FGF23)-klotho axis and early vascular aging (EVA). Main effectors of the phosphate homeostasis with potential effects on EVA components. In the healthy vasculature (upper half of the vessel), endothelial cells and vascular smooth muscle cells (VSMC) surround the arterial lumen. Phosphate accumulates in the circulation of patients with advanced chronic kidney disease (CKD) leading to VSMC and endothelial dysfunction (lower half of the vessel). Bone-secreted FGF23 also adversely (highlighted in *red*) associates with EVA. In contrast, the antiaging-associated klotho beneficially (highlighted in *green*) counteracts EVA, cellular senescence, inflammation, and oxidative stress. The role of vitamin D analogs on EVA is not clear, so far, and both beneficial and negative studies have been published.

Furthermore, a few experimental studies link FGF23 to ED [116,117]. Importantly, FGF23 neutralization resulted in higher mortality and a dose-dependent increase in serum phosphate and aortic calcification in a rat model of CKD-mineral and bone disorder [118] further questioning whether FGF23 has an effect on EVA.

Regarding "whole body" aging, as well as EVA, klotho is the most promising member of endocrine phosphate-FGF23-klotho axis (Fig. 20.1), as Kuro-o et al. [99] described a syndrome resembling human aging in klotho-deficient mice. Recently, klotho-deficient zebrafish also show shortened lifespan and increased VC accompanied by activated osteoclast differentiation pathways confirming the mice data [119]. When renal function declines, klotho levels decrease in humans and in rodents [99]. Conversely, klotho-overexpressing mice with CKD have an increased phosphaturia, an improved renal function and, most importantly, a significantly lower calcification in the kidneys and aortas indicating an improved EVA phenotype [100,120]. Moreover, a recent study relates cellular senescence with a downregulation of klotho [121]. In more detail, klotho prevents the osteogenic phenotype switch of VSMC in aortas of CKD mice, as well as the phosphate influx in rat VSMC in vitro [120]. Interestingly, both high phosphate and inflammation also decrease renal *Kl* mRNA expression in vitro [122] suggesting that klotho deficiency observed in CKD could be a potential pharmacological target for the treatment of VC. Besides VC, klotho also exerts beneficial effects on the endothelium. Thus, klotho attenuates increased expression of inflammatory and adhesion molecules, as well as augments NO production and vasorelaxation [123–125]. Moreover, klotho suppresses nuclear factor NFκB activation and the subsequent transcription of proinflammatory genes [126], and klotho may also exert some of its beneficial effects on EVA by activating the NRF2 pathway [127]. In conclusion, most if not all members of the endocrine phosphate-FGF-23-klotho axis have effects on EVA and translationally, klotho stands out as a potential pharmacological target for the prevention and/or treatment of EVA in CKD.

The NRF2-KEAP1 axis and early vascular aging

The NRF2-kelch-like ECH-associated protein 1 (KEAP1) axis is a major defense system regulating antioxidative and antiinflammatory genes providing cytoprotection against different pathologies [128,129]. In the presence of cellular (e.g., oxidative) stress, NRF2 is released from its repressor KEAP1, translocates to the nucleus, and acts as transcription factor regulating the expression of a plethora of genes involved in cellular stress and damage repair [128,130] that have an antioxidant response element in their promoter regions [129]. Importantly, NRF2 expression is low in different lifestyle diseases, including respiratory (e.g., smoking-related lung emphysema), gastrointestinal (e.g., primary biliary cholangitis), metabolic (e.g., insulin resistance and nonalcoholic steatohepatitis), and neurodegenerative (e.g., Alzheimer's and Huntington disease), as well as CKD [128,129,131]. NRF2 is also inversely associated with a distinct rare progeroid syndrome, i.e., Hutchinson-Gilford progeria syndrome (HGPS), with extreme EVA and cardiovascular death as stroke and myocardial infarction during teenage [128]. Thus, HGPS is caused by mutations in lamin A-coding *LMNA* gene [132]. Mechanistically, patients with HGPS express a lamin A mutant known as progerin which directly impairs NRF2 activity [133]. Reactivation of NRF2 in ex vivo cells of patients with HGPS can reverse aging defects [133] indicating that NRF2 is directly and closely linked to aging. In the animal kingdom, upregulated NRF2 expression seems to be associated with a survival advantage in animals living in extreme environments [128].

Regarding EVA, Ungvari et al. [134] have demonstrated an age-dependent NRF2 dysfunction in cultured rat aortas in accordance with findings from progeroid syndromes. Data from the same group confirm increased cellular senescence and SASP in cerebral arteries of NRF2-deficient rats compared to control animals [135]. Conversely, miRNA secreted from senescent endothelial cells decreases NRF2 expression resulting in reduced stress tolerance [136] suggesting an autocrine, senescence-dependent effect on NRF2 in the vasculature. Similar findings (i.e., NRF2 is linked to markers of cellular senescence) have also been reported in VSMC in which NRF2 activity lowered angiotensin II-induced markers of cellular senescence [137]. Taken together, as silencing of the NRF2 gene promote premature senescence [138] there seems to exist a bidirectional association between NRF2 expression and cellular senescence. Similar to klotho and besides the above-described effects on vascular cellular senescence, NRF2 has also been linked to both major components of EVA, i.e., VC and ED. Exposure of VSMC to high phosphate increases ROS and NRF2 activation in vitro [139]. Since FGF23 was shown to activate NRF2 signaling cascades to protect osteoblasts from oxidative injury and cell death [140], the effects of FGF23 on vascular NRF2 expression deserve attention.

The causal role of NRF2 on oxidative stress defense and calcification was demonstrated in NRF2 silencing, as well as NRF2 activation by tertiary butylhydroquinone [139]. In further in vitro experiments on VC using high phosphate-treated VSMC, NRF2 overexpression decreased mRNA expression of *Runx2* and bone morphogenetic protein 2 compared to control cells suggesting a sustained contractile phenotype [141]. Furthermore, Yao et al. [141] show increased autophagy markers in NRF2-overexpressing VSMC. NRF2 is also involved in the effect of several

compounds on VC. Thus, the antioxidant mitoquinone decreases calcification of VSMC through the NRF2/KEAP1 axis, and the effects were lost when cells were additionally treated with an NRF2 inhibitor [142]. Hydrogen sulfide (H_2S) is an important, gaseous signaling molecule exerting beneficial effects on endothelial cells and VSMC [143], which at least partly depends on NRF2 activation. Thus, H_2S attenuates calcification of VSMC via activation of the NRF2-KEAP1 axis [144].

In contrast to VC, data on NRF2's effects on ED are scarce. Some evidence is available in sheer stress-related ED [145]. Thus, Takabe et al. [145] reported that endothelial cells under sheer stress express adverse adhesion molecules and chemokines especially when NRF2 was silenced, and they propose that NRF2 is an inducer of an antiatherogenic environment. Taken together, the NRF2/KEAP1 axis is a promising translational target for the prevention and/or treatment of EVA in CKD.

Holistic therapeutical approaches for early vascular aging in chronic kidney disease

Despite considerable efforts, the cardiovascular morbidity and mortality of patients with CKD, and especially ESKD, are still very high [1–3]. However, recent data from the ERA-EDTA registry are encouraging since it shows that absolute case-specific excess mortality due to atheromatous CVD tends to decrease from 2007 to 2015 in patients on RRT [146]. It is interesting to note in this context that in contrast to non-CKD populations, established treatment approaches for cardiovascular risk factors are not successful in patients with ESKD. For instance, statin treatment improving a proatherogenic lipid profile showed disappointing results in dialysis patients [13,14]. Furthermore, pharmacological lowering of the adverse hyperphosphatemia in patients with CKD does not seem to lower VC/EVA [147]. Moreover, the oral charcoal adsorbent AST-120 lowers specific uremic toxins and dose-dependently decreases circulating levels of the adverse uremic toxin IS [148]. However, results of clinical trials on AST-120 were heterogeneous and there was no effect of adding AST-120 to standard therapy in patients with moderate--severe CKD on a primary composite renal end point [149]. Thus, novel and additional therapeutical approaches for the two major EVA components, i.e., VC and ED, are needed. A novel potential treatment approach should show the following characteristics: It should (1) be convenient to administer, i.e., not requiring large amounts of (intravenous) fluids to dissolve due to fluid intake restriction in ESKD; (2) have stable gastrointestinal uptake due to gut dysbiosis in CKD [150]; (3) should holistically improve VC, ED, and finally reduce major adverse cardiovascular events and mortality; and (4) exert only minor side effects in the highly vulnerable CKD population.

Klotho—Could it be targeted?

Kidney-derived klotho is of particular interest as a potential treatment for EVA but also whole body aging in CKD as klotho-deficient mice and patients with CKD have similar phenotypes, e.g., increased VC [120], ED [151], a proinflammatory status [152,153], and SASP [152]. In contrast to other cardiometabolic diseases in which a resistance has been described for a candidate (e.g., insulin resistance in patients with type 2 diabetes), patients and animals with CKD show decreased protein expression of klotho in the plasma, urine, and the kidneys [120]. Thus, klotho appears to be an ideal candidate for the prevention and/or treatment of EVA and would allow simple treat-to-target strategies.

In general, therapeutic approaches to stimulate klotho expression can either increase endogenous klotho indirectly or directly administer exogenous klotho, and have recently been reviewed elsewhere [100,154]. Importantly, all of these approaches increase klotho protein systemically but not specifically in the vasculature. To raise endogenous klotho levels that are diminished in the uremic milieu, different drug classes have already been tested and encouraging results have been reported. However, the vast majority of studies have used animal experiments and human data on klotho inducers are scarce. In rats, the insulin-sensitizing thiazolidinedione pioglitazone protects against renal injury in aging by an increased expression of klotho [155]. However, there are no data so far from human studies using thiazolidinediones on klotho levels. Furthermore, the vitamin D analog paricalcitol induces the expression of klotho in the kidneys of uremic rats [156]. In accordance, mice with CKD treated with paricalcitol or calcitriol also show increased serum and urinary klotho levels [157]. Importantly, both studies did not find increased vascular klotho expression [156,157] limiting the potential of vitamin D analogs for the treatment of EVA. In vitro data using murine internal medulla collecting duct epithelial cells suggest that statins induce *klotho* mRNA expression [158]. However, no study has investigated whether statin treatment increased klotho expression in the vasculature for the potential and specific EVA treatment. Moreover, since angiotensin II decreases *klotho* mRNA expression in vitro [158], the angiotensin II type 1 receptor blocker losartan reverses the decrease in *klotho* expression in mice

with cyclosporine-induced renal injury [159], as well as in rats with diabetic kidney disease [160]. Testosterone may also increase renal klotho gene expression via the androgen receptor-mediated pathway [161]. However, results from a randomized, double-blinded, placebo-controlled study do not find altered soluble klotho levels after 6 months of testosterone therapy [162]. In contrast to secondarily and indirectly increased klotho levels by other drugs, direct administration of exogenous, soluble klotho has also been proven to be effective for increasing circulating klotho levels, as well as protecting against acute kidney injury [163] and CKD [154]. Furthermore, direct, recombinant, human klotho treatment has been shown to be effective in ameliorating the activated renin-angiotensin system and reducing blood pressure in mice with polycystic kidney disease [164]. Using a plasmid vector encoding full-length mouse klotho, the role of klotho on salt-sensitive hypertension has also been validated recently [165]. Since the NRF2 agonist sulforaphane (SFN) and klotho both enhance antioxidant defenses that protect against VSMC dysfunction in CVD [166] combinations of NRF2 agonists and klotho may have complementary effects.

Importantly, klotho is downregulated in several cancer types and is also recognized as a future potential antitumor therapy [167] suggesting that klotho treatment is theoretically feasible for many patients. Despite these data favoring especially direct klotho supplementation for prevention and/or treatment of EVA, some caveats have to be taken into consideration [168]. Firstly, there are several isoforms of klotho and it is not known, so far, which isoform and which klotho domain is most relevant in EVA. Secondly, when using a treat-to-target approach for klotho dosing, circulating klotho levels need to be quantified in a reliable and specific manner which is not possible [168], so far. Thirdly, the half-life of exogenous klotho depends on renal function which needs to be taken into account for drug dosing [169]. In conclusion and based on the discussed first in vitro and in vivo, as well as safety, data with regard to cancer, especially direct klotho treatment appears to be an attractive future treatment option for EVA and CKD.

NRF2—A cytoprotective transcription factor that can be targeted by lifestyle factors

The NRF2/KEAP1 system is a key regulator of cytoprotection against cellular stress promoting NRF2 as a multiorgan protector. Concluding the above-discussed findings, treatment of the repressed NRF2 system in CKD may improve premature aging [128] and in particular EVA [127,135]. NRF2-inducing treatment options include nutritional components [128], exercise [170], and synthetic agonists which target NRF2 or inhibit the binding of NRF2 to KEAP1 [129,171]. In a screening of 54 natural compounds on their effects on antioxidant response element promoters, several compounds had a significant effect on NRF2-mediated antioxidant response element promoter expression [172]. Indeed, bioactive foods, such as broccoli sprouts, tomato skin, turmeric, red-purple plants, and coffee beans, are nutritional stimulators of NRF2 expression that could improve the inflammatory, senescent, and pro-oxidative uremic phenotype [171]. In more detail, seven out of the 54 natural compounds had an even higher effect compared to *tert*-butylhydroquinone [172], i.e., a prototypical NRF2 activator [173]. Mafra et al. [171] have recently summarized the current knowledge on natural compounds on NRF2 activation, and different compounds were ranked based on their efficacy [128], proposing that "Food as medicine" could be a novel option for targeted treatment of EVA in the uremic milieu.

Andrographolide (ranking first based on its NRF2-activating efficacy [128,172]) is the main bioactive component of the medicinal plant *Andrographis paniculate* and has been reported to NRF2-dependently decrease proinflammatory and vasoconstricting endothelin 1 expression in human endothelial cells [174]. Furthermore, andrographolide suppresses TNF-induced ICAM-1 expression in HUVECs [175] (Fig. 20.2). In contrast to andrographolide, no data on the NRF2-dependent effects of *trans*-chalcone on the components of EVA have been reported. Sulforaphane (SFN) is a natural compound found in broccoli sprout and other cruciferous vegetables that exerts metabolically beneficial effects [176]. SFN suppressed an angiotensin II-induced VSMC phenotypic switching and proliferation [177] supporting previous findings [178]. Furthermore, SFN reduced TNF-mediated HUVEC secretion of endothelin 1, VCAM1, ICAM1, and E-selectin [179], and prevented increased endothelial permeability [179], as well as arterial inflammation [180]. Moreover, treatment with SNF (provided as concentrated broccoli sprout extract) activates NRF2 and reduces ROS production [181] and has epigenetic effects that modulate proteasomal function and mitochondrial dynamics [182]. It was recently reported that SFN prevents age-associated cardiac and muscular dysfunction through NRF2 activation [183]. Similarly, for curcumin, an extract from the turmeric plant, an NRF2-dependent induction of heme oxygenase-1 expression was shown resulting in lower vascular inflammation [184]. Furthermore, curcumin also inhibits endothelial-to-mesenchymal transition and endothelial fibrosis in vitro [185] (Fig. 20.2). Flavonoids (e.g., from plant extracts rich in flavan-3-ols) have been linked to increased activity of NO synthase in endothelial cells [186]. Importantly, these findings have been validated in HD patients and a cocoa flavanol beverage lead to an improved endothelial function [187] (Fig. 20.2). Epigallocatechin-3-gallate, another nutritional NRF2 agonist and the major component of green tea, has also been associated with EVA and reduces the proliferation of VSMC in vitro [188], induces NO-dependent vasodilation [189], NO bioavailability [190], and reduces endothelial inflammation [191] (Fig. 20.2).

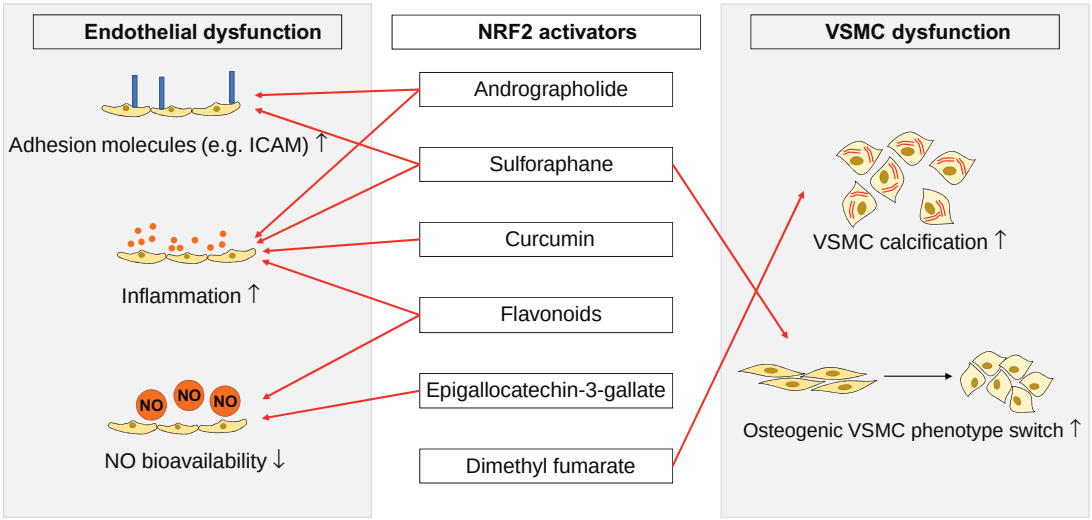

FIG. 20.2 Mechanisms by which nuclear factor-erythroid 2 (NRF2) agonists improve early vascular aging (EVA). Different NRF2 agonists have been causally linked to components of EVA, i.e., endothelial dysfunction (*left*, *gray panel*) and VSMC dysfunction (*right*, *gray panel*). *Red lines* indicate by which mechanism the respective NRF2 agonists reduce EVA. Only a selected number of pathophysiological mechanisms between NRF2 agonists and EVA are shown. *ICAM*, intercellular adhesion molecule 1; *NO*, nitric oxide; *VSMC*, vascular smooth muscle cells.

In the group of synthetic NRF2 inducers, bardoxolone and the rodent-tolerable bardoxolone methyl analog dihydro-CDDO-trifluoroethyl are among the most promising pharmacological candidates with positive renal outcome data in a phase 2 trial in patients with type 2 diabetes and advanced CKD [192]. However, previous treatment approaches for patients with diabetic kidney disease (DKD) using bardoxolone have been terminated due to an excess of heart failure hospitalizations in the bardoxolone group [193]. Interestingly, another NRF2 analog has already shown that the NRF2 activator dh404 attenuated endothelial dysfunction in diabetes [194]. Furthermore, bardoxolone was the most potent NRF2 activator among three tested compounds, and showed a reduced endothelin 1 release [195]. Moreover, dimethyl fumarate (DMF) dose-dependently inhibited VSMC calcification [196] indicating a class effect of all members of NRF2-activating drugs.

Despite the many beneficial effects of NRF2 agonists, potential caveats must also be acknowledged: Firstly, NRF2 has dual roles in its association with carcinogenesis, as well as cancer progression and therapy. Thus, NRF2 activation in normal cells can prevent cancer initiation [197]. In contrast, prolonged NRF2 activation is involved in cancer promotion, progression, and treatment resistance [197]. Consequently, each NRF2-based approach must carefully investigate oncogenic risk as a potential side effect. Secondly, despite promising initial results, previous treatment approaches for patients with DKD using the NRF2 agonist bardoxolone have been stopped due to an excess of heart failure hospitalizations among those assigned to bardoxolone [128]. However, since patients with risk factors for heart failure could be identified and excluded, bardoxolone is currently being re-investigated in different clinical trials comprising patients with CKD and differing underlying pathologies, such as Alport syndrome and adult polycystic kidney disease [129]. When patients in these studies are carefully selected, the NRF2 agonist might have beneficial effects on the EVA components, e.g., ED and VC. Thus, NRF2 is currently a promising and clinically advanced signaling pathway for a possible future prevention and/or treatment of EVA in CKD.

Could senolytics arrest early vascular aging in chronic kidney disease?

Senolytic drugs extend lifespan and delay age-related diseases in mice by specifically targeting and eliminating senescent cells [82]. However, since selectivity against senescent cells is modest and cell-type specific, further research is needed until treatment could be initiated in humans. As senescence is related to repressed activation of the antioxidative transcription factor NRF2, this opens additional possibilities for treatment. As mentioned above, senescent cells generate a proinflammatory secretome (SASP) that results in a bystander effect and promotes local tissue inflammation that poison the tissue and impair its functions. Emerging evidence indicates that many nutrients exert their effects through modulation of factors that regulate inflammation [198] and/or telomere attrition [199]. As advanced CKD [200] and EVA with VC [81] are characterized by cellular senescence, treatment with senotherapeutics including senolytic drugs (i.e., therapeutic agents that induce apoptosis of senescent cells), such as dasatinab [201], may be of major interest to study in CKD. The efficacy of senolytics requires synergistic use of another senolytic

agent, such as quercetin or fisetin, that enhances the senescent cell specificity of the apoptotic effect [202]. Thus, treatment with dasatinab needs to be combined with another senolytic agent. Since quercetin and other natural senolytic compounds, such as resveratrol and fisetin, that can be acquired nutritionally also increase NRF2 activation, tailored nutritional interventions using senolytics for the high-risk vascular phenotype in CKD should be performed.

Conclusions

The concept of vascular senescence and EVA is an important risk factor for the development of premature CVD in patients with CKD. Different pathophysiological mechanisms frequently present in patients with advanced CKD account for the increased vascular senescence and EVA, including uremic toxins, cellular senescence, inflammation and oxidative stress, klotho deficiency, and repressed NRF2. Potential holistic approaches for prevention and treatment of vascular senescence and EVA in CKD include klotho and NRF2 activation, as well as senotherapeutics. Klotho and the NRF2/KEAP1 signaling pathway are currently the most promising candidates and will, therefore, be of major interest to reduce the burden of disease due to CKD.

Declaration of interest

P.S. serves on Scientific Advisory Boards of Baxter, Astra Zeneca and REATA. T.E. has served as a consultant for Sanofi-Aventis, as well as on a scientific advisory board of Boehringer Ingelheim Pharma.

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Author contributions

T.E. and P.S. wrote the manuscript and researched literature.

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CHAPTER

21

Injury and regeneration in renal aging

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Senescence begins
And middle-age ends
The day your descendants
Outnumber your friends
—Ogden Nash

Introduction

For the past 100 years, global human life expectancy has doubled, mainly due to the success of fighting infectious diseases. Such expansion in lifespan has changed the definition of an aged individual. Today, the onset of "old age" in humans is set between 60 and 65 in the developed countries, partly linked to the age of retirement [1]. However, despite the continuous progress in health care, life expectancy has plateaued in developed countries in recent years [2]. Simultaneously, the proportion of "aged" individuals (> 65 years) is expected to rise by 40% by 2040. The increase in the aged population is expected to pose a serious burden to healthcare systems worldwide and warrants further research on aging-related diseases.

Although the aging process is ubiquitous and inevitable, it is far from being understood [3]. Aging is a multifactorial process defined as age-dependent, progressive decline in intrinsic function of body organs due to impaired homeostatic and adaptive capabilities, leaving an individual more susceptible to environmental stress [4, 5]. The evolutionary purpose of aging is a matter of ongoing debate. Aging was previously widely accepted as a genetically programmed process, but the current view of aging sees it as a combination of genetics, environmental factors, and chance [6]. Supporting evidence for this new perspective includes different aging patterns observed in monozygous twins and low heritability of lifespan [7, 8].

We discuss the classic manifestations of kidney aging from a cell-specific view point, and then review possible approaches to alter the dysregulated cell mechanisms that occur in aging and promote regeneration of aging kidneys.

Injury of aging kidney

Systemic vs renal aging

Aging takes place in every organ, but its rate between organs differs [9]. For example, while musculoskeletal and gastrointestinal systems seem to be more resilient to aging, cardiovascular, pulmonary, endocrine, and renal systems show more profound age-related decline [9]. In addition, only 19%–26% of age-associated genes overlap between whole blood and brain tissue as assessed by a whole-genome transcriptomic analysis [10]. Further, age-associated genes vary by 30%–60% between human races. Still, the systemic aging "milieu" is essential for aging to occur, as demonstrated by experiments in rodents using parabiosis [11]. Blood from young mice was able to prolong the life

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of older mice, and in turn, young mice receiving blood from old mice showed signs of premature senescence [12]. Similarly, transfer of gut microbiota from young-to-old African turquoise killifish, a vertebrate with a short lifespan (4–8 months), prolonged the lifespan in the old fish [13].

Cell senescence is recognized as the key characteristic of aging. There are multiple factors contributing to cell senescence, including genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, stem cell exhaustion, and altered intercellular communication [14]. Animal models, especially rodents, have provided useful insight into aging, and genetic or pharmacologic manipulation of various molecules (e.g., Klotho, insulin-like growth factor (IGF-1), and mechanistic target of rapamycin (mTOR)) has significantly increased the lifespan of mice [6]. However, rodent models of aging have their limitations, and translation to human research is not always straightforward. For example, the telomeres in mice are 5–10 times longer than in humans, and telomerase enzyme deficiency in mice did not induce premature senescence as observed in humans [15]. The kidney is a complex organ, comprising more than 20 different cells [16]. These include several types of highly specialized epithelial cells, endothelial cells, mesenchymal cells, and resident immune cells. This heterogeneity is reflected in the way a kidney ages and regenerates.

Renal aging

The surface of an aged kidney is rougher and darker, and the kidney is smaller than a young adult kidney [17]. Reduction of renal mass and loss of functional nephrons are hallmarks of the aging kidney [18]. The overall kidney volume shows a slight increase with medulla increasing slightly more than cortex, mostly in males, during the second and third decades of life, followed by a progressive decline of cortical mass starting at the fourth decade of life [19]. More recent data indicate that kidney mass increase might extend to the fourth to fifth decade of life [20]. There was a progressive decline of kidney mass in women and accelerated decline after the fifth decade of life in men. The number and size of renal cysts increase with age, mainly in the cortical region, more prominently in males vs females [21]. Even in healthy aged individuals, simple renal cysts and other benign renal masses increase with age [22].

The total number of nephrons per kidney determines its functional capacity [23]. In humans, nephrogenesis is completed at the time of birth, with an average number of ~ 1 million nephrons per kidney [24], and with a high interindividual variability already at birth (15 infants; nephron number ranging from 250,000 to 1.1 million) [25]. Variability was even higher among adults, ranging from 210,000 to 2.7 million nephrons per kidney [25–27]. The number of nephrons declines progressively during aging, with a loss of ~ 7% of nephrons per decade, corresponding to ~ 50% of nephron loss from age 18 to 75 years [23]. Low birth weight is associated with lower nephron number per kidney even at birth and may subsequently also be related to accelerated nephron loss during aging [28, 29].

When nephrons are lost, those remaining hypertrophy. Glomerular volume increased with aging in relatively small autopsy studies [26, 30, 31]. Of note, possible associated comorbidities were not always excluded [32]. In fact, studies in normal aging performed in a large number of biopsies (~ 1400–1500) of healthy living kidney donors at different ages did not find glomerular enlargement with aging [17, 33]. On the contrary, tubular volume increased with age and was considered responsible for aging-related nephron hypertrophy and kidney volume increase, not surprising, given that tubules represent ~ 90% of the kidney volume. Of interest, glomerular density was less in areas of biopsies with less nephrosclerosis, apparently reflecting less nephron loss.

The morphological changes of aging are accompanied by a decline in glomerular filtration [18]. After the fourth decade of life, the glomerular filtration drops by 8 mL/min/1.73 m^2 every 10 years [34]. The rate of the decline likely accelerates with increasing age [35]. However, several studies were unable to directly correlate the reduction of renal mass and age-related decrease in glomerular filtration [36]. Moreover, nephron hypertrophy and nephrosclerosis in healthy aging do not correlate with glomerular filtration [17]. Age-related tubular dysfunction includes decreased urine dilution and concentration capacity, Na^+ reabsorption, and K^+ transtubular transport [37].

The aging kidney morphologically shows increased nephrosclerosis, including arteriosclerosis, focal and global glomerulosclerosis (GS), glomerular basement membrane (GBM) thickening, tubular atrophy and dilation, and interstitial fibrosis [38]. Vascular alterations of the aging kidney include increased intimal proliferation in arterioles, increased intrarenal shunting and capillary bypassing, and increased extracellular matrix (ECM) deposition [37]. In normal aging, only focal and global GS develops, and the presence of segmental GS is a sign of accompanying kidney disease. Ischemia due to arteriosclerosis and hyalinosis of small arteries is likely the underlying mechanism contributing to nephrosclerosis. Ischemic glomeruli show wrinkled capillary tufts, GBM thickening, pericapsular fibrosis followed by collagen deposition within the Bowmańs space, and a collapse of the glomerular tuft, the so-called obsolescent pattern of global glomerulosclerosis [39, 40]. Involution of such globally sclerosed glomeruli is common [41]. GS in aging can also be triggered by a progressive loss of podocytes [17]. Tubules connected to the sclerosed

glomeruli atrophy, and interstitial fibrosis develops [42]. In addition, tubular derangements may result in increased atubular glomeruli, i.e., glomeruli without connection to the proximal tubule. An overview of the functional and structural changes of an aging kidney is shown in Fig. 21.1.

Apart from "healthy" aging described earlier, the aging process renders the kidney more susceptible to any additional injury. Aging is a risk factor for developing both acute (AKI) and chronic kidney injury (CKD), as well as renal cancer [43].

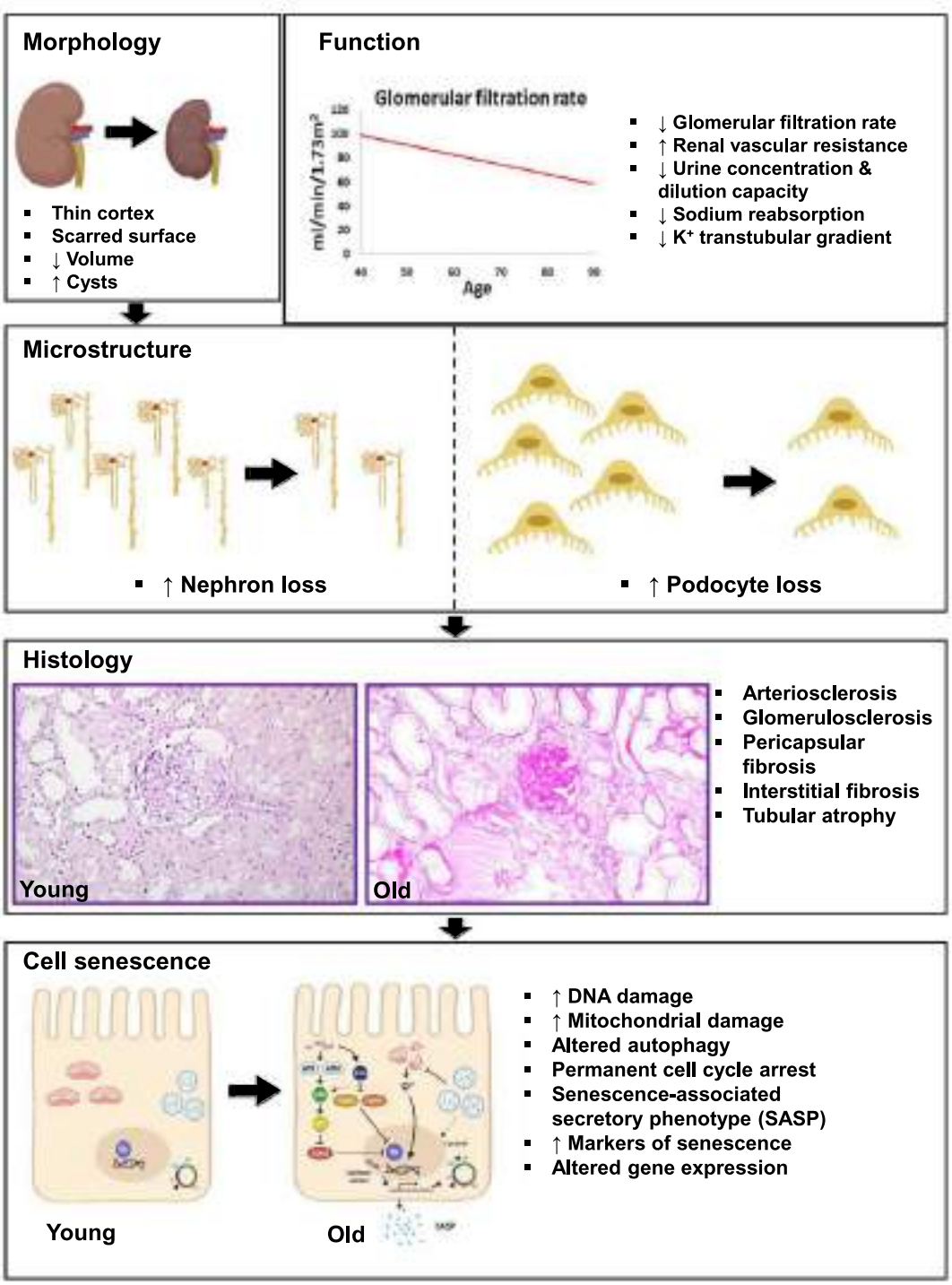

FIG. 21.1 Characteristics of the aging kidney.
Changes in the aging kidney can be seen on several different levels. Functional changes include a decline in glomerular filtration (approximately 8 mL/min/1.73 m² per decade of life), increased renal vascular resistance, and tubular dysfunction. Structural changes are reflected in both overall morphology and microstructure. Cell senescence is the key feature of aging kidney on the cellular level.

The mechanisms contributing to aging and injury show both unique and shared traits, and as such, they are often studied in parallel (see also Chapters 3 and 4). In this chapter, we primarily focus on aging; however, due to a paucity of data in some areas of aging, parallels to kidney injury are drawn.

Mechanisms of aging

Cell senescence

Cell senescence is a state when a cell is no longer able to replicate due to cell cycle arrest [44]. Senescent cells are characterized by altered phenotypes, including increased heterogeneity, chromatin rearrangements, autophagy modulation, expression of senescence-associated β galactosidase, accumulation of lipofuscin granules, and lack of response to mitotic stimuli [45]. The secretome of these cells changes to the so-called senescence-associated secretory phenotype (SASP), consisting of cytokines, growth factors, and extracellular proteases, promoting inflammatory signaling [46]. It should, however, be emphasized that senescent cells are still viable and functional and have important roles in crucial physiological functions, such as tissue remodeling, stress response, embryonic programming, and limiting precancerous cell proliferation [46].

Cell senescence induced by DNA alterations

DNA replication and damage play a crucial role in the cell's shift toward the senescent phenotype. The number of cell cycles a cell can undergo is defined by an equilibrium between shortening and lengthening of telomeres, repetitive sequences of DNA, and telomere-binding proteins, located at the end of chromosomes [47]. Telomeres shorten with every cell replication and subsequently become elongated by telomerase, an RNA-dependent DNA polymerase. When the equilibrium shifts toward telomere shortening, the telomeres become too short for a safe replication cycle, the DNA damage signal is recognized, cell cycle arrest is triggered, and replicative senescence occurs.

Cell senescence can also occur via so-called stress-induced premature senescence (SIPS), when DNA damage is triggered by oxidative stress, mitochondrial damage, activation of oncogenes, cell fusion, epigenetic stress, or SASP signals from previously senescent cells [48–51]. Oxidative damage can also induce direct telomere damage, resulting in cell senescence [52].

To date, several different pathways involved in cell senescence have been described [53]. The DNA damage response (DDR) pathway relies on ataxia-telangiectasia mutated (ATM) and ATR (ATM and Rad3 related) kinases, which stabilize p53 and activate cyclin-dependent kinase inhibitor p21, which inhibits Cdk2 (cyclin-dependent kinase) and induces cell cycle arrest. This pathway is mainly responsible for recognizing telomere attrition, oxidative stress-induced DNA damage, and activated oncogenes. DDR-independent pathways are involved in senescence triggered by activation of E2F2 or inhibition of c-Myc and involve $p16^{Ink4a}$ or $p19^{Arf}$ [53]. $p16^{Ink4a}$ activates Cdk4 and Cdk6 to induce cell cycle arrest. Retinoblastoma (Rb) is the common target for both p53 and $p16^{Ink4a}$, which they stabilize to prevent activation of the transcription factor E2F2. The two pathways can either act in unison or antagonize each other.

In addition to different triggers of cell senescence, the development of cell senescence is a multistep process [53], evolving over time. To better describe the process, a parallel to terminally differentiated cells can be drawn. Terminally differentiated cells are in a so-called quiescent state or transient cell cycle arrest, and they do not proliferate. However, compared with senescent cells, their proliferative capacity can be restored after certain stimuli, e.g., injury. Early senescence is defined by a shift from transient to permanent cell cycle arrest by DDR-dependent or DDR-independent pathway signaling. Subsequent downregulation of lamin B1, a nuclear membrane protein, results in chromatin rearrangements and induction of SASP, resulting in late senescence [53]. The senescent cells are either recognized by the immune system or progress to the final third state of deep senescence, characterized by chromatin budding, histone proteolysis/replacement, retrotransposition, and additional changes in SASP.

The specific triggers of cell senescence also influence its kinetics. Acute senescence is considered a beneficial injury response in wound healing, injury response, and embryonic development, and these cells are eliminated by the immune cells. On the contrary, chronic senescence is likely the result of sustained stress and damage, leading to the accumulation of senescent cells and their impaired elimination by immune cells. The latter is often observed in aging and is considered central to aging theories based on organ accumulation of senescent cells. A pool of mixed senescent cells is often observed in cancer, where acute senescence may shift into chronic senescence.

Autophagy in cell senescence

Autophagy is a process responsible for the removal of damaged organelles and altered proteins, thus regulating cell homeostasis and stress response [54]. It occurs via formation of autophagosomes that engulf and transport molecular cargo marked for degradation, and eventually fuse with lysosomes, where the degradation occurs. Yet,

autophagy is not only a simple degradation process. In the past two decades, an enormous effort in autophagy research has uncovered its roles in various processes, including cell senescence. However, its role in cell senescence is not straightforward, as it can both inhibit and induce cell senescence. Under stress, autophagy can facilitate prosurvival signaling in cells. Lack of nutrients induces changes in several pathways, including the mechanistic (also called mammalian) target of rapamycin pathway (mTOR), sirtuin 1 (Sirt-1), and adenosine monophosphate-activated protein kinase (AMPK) [55]. mTOR is inhibited in nutrition-free conditions. A degradation of cytosolic components, which then serve as a source of energy for the cell, is triggered. Genetic mutation or pharmacologic inhibition of mTOR increased lifespan in different model organisms, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and also in mice [56]. Caloric restriction (CR) by reduced food intake is a strong inducer of autophagy and also prolonged lifespan in some, but not all, studies in a variety of species, including rhesus monkeys [57, 58]. Still, epidemiological studies suggest many beneficial effects of CR on human health, e.g., decline in blood pressure, insulin sensitivity index, and LDL cholesterol [59].

Autophagy is also essential in the maintenance of homeostasis of organelles, especially mitochondria. Accumulation of damaged or dysfunctional mitochondria is a source of oxidative stress. CR increases Sirt1 expression, which induces autophagy and attenuates hypoxia-induced mitochondrial damage [60]. On the contrary, autophagy was found to promote cell senescence by a negative feedback loop on the mTOR pathway [61].

Circulating factors in aging

Klotho, Wnt signaling, and the renin-angiotensin-aldosterone system (RAAS) all have circulating elements that can influence aging. Klotho, known as the antiaging molecule, is a transmembrane protein resembling β-glucuronidases [62]. It is mostly produced by the kidney and expressed mainly on the tubular epithelium [62]. The extracellular domain of Klotho can be cleaved by secretases, be released to the circulation, and act as a hormone [63]. Systemic overexpression of Klotho increases lifespan, and exogenously added Klotho reduces endothelial cell senescence in mice [64, 65]. Klotho regulates several pathways involved in cell senescence. It inhibits IGF-1/insulin signaling via IGF-related receptor 1. This is an evolutionarily conserved pathway whose inhibition prolongs lifespan among different species by regulating metabolism, feeding behavior, reproduction, and resistance to oxidative stress [66]. Klotho also inhibits the evolutionarily conserved Wnt/β-catenin signaling pathway. Loss of Klotho led to upregulation of Wnt/β-catenin and exacerbation of kidney injury in mice [67]. In turn, Klotho-mediated inhibition of the Wnt/β-catenin pathway reduced cell senescence by decreasing age-related mitochondrial damage via the RAAS [68]. Wnt family gene expression, including those involved in the noncanonical Wnt signaling, was upregulated in the aging kidney. Further, Wnt9a accelerated cell senescence in tubular cells following ischemia-reperfusion injury [69]. Wnt5a was not overexpressed in the aging kidney in these studies. However, in Klotho-deficient aged mice, Wnt5a was increased in the aorta and promoted high salt-induced hypertension [70]. In addition, Wnt5a regulated angiotensin II (Ang II)-induced reduction in renal blood flow, which was reversed by Klotho. Interestingly, the lifespan of mice deficient in Ang II type 1a receptor (AT1a) increased by ~ 26%, which attributed mainly to reduced oxidative stress, a higher number of mitochondria, and less atherosclerosis in the aorta [71]. Whether this phenotype was contributed to by changes in Wnt or Klotho is not known. These findings show complex interactions of the RAAS, Klotho, and Wnt signaling, which are modulated by salt during aging. A schematic overview summarizing the mechanisms and pathways contributing to cell senescence is shown in Fig. 21.2.

Cell-specific aspects of renal aging

In the aging human kidney, both telomere shortening and cell senescence markers are present [72]. The CDK inhibitor $p16^{INK14a}$ increased with age with cell-specific heterogeneity. In the glomerulus, $p16^{INK14a}$ was expressed on podocytes and parietal epithelial cells (PECs). The highest staining intensity was found on distal tubules and macula densa cells, and less staining was observed in proximal tubules and collecting ducts. Peritubular capillaries and vascular smooth muscle cells of arteries also showed positive staining. Similar increases in $p16^{INK14a}$ were seen in rodent kidneys, even without telomere shortening [73]. p27, another CDK inhibitor, was also increased in aged human kidneys in most cell types [74].

Senescence of glomerular epithelial cells

The number of podocytes per glomerulus in healthy adult humans has a relatively wide range (263–983) and increases by about 20% from adolescence to adulthood [75]. At the same time, podocytes are continuously lost during a normal lifespan, with podocyturia detected as early as 3 years of age in healthy children [76]. Podocyte loss is further accelerated with increasing age or in the presence of kidney disease [77, 78]. Podocytes isolated from urine of healthy

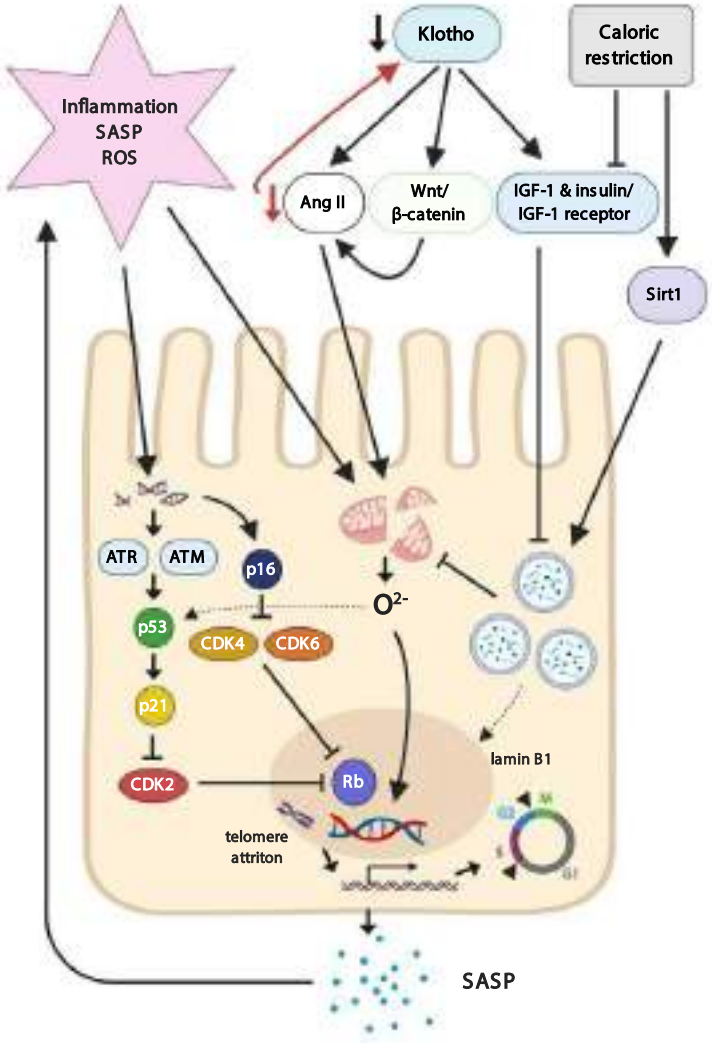

FIG. 21.2 Mechanisms of renal aging.
A complex interplay between circulating factors and cell compartments influences the development of cell senescence. DNA damage, whether a result of telomere attrition or external stimuli, such as inflammation, reactive oxygen species (ROS), or senescence-associated secretory phenotype (SASP), is recognized by multiple pathways able to trigger cell cycle arrest. Mitochondrial damage generates ROS, which might either directly induce damage DNA or activate a pathway responsible for its recognition. Autophagy eliminates ROS by removal of damaged mitochondria but can also contribute to cell senescence by degrading lamin B1 in the nuclear membrane, leading to destabilization of the nuclear contents.
A decrease in the antiaging molecule, Klotho, leads to increase of Ang II, Wnt/β-catenin signaling, and IGF-1/insulin signaling via IGF-1 receptor. Ang II increases ROS by mitochondrial damage. In turn, decrease in Ang II can upregulate Klotho. Wnt/β-catenin activates Ang II. IGF-1/insulin signaling inhibits autophagy. On the contrary, caloric restriction is one of the strongest inducers of autophagy by inhibition of IGF-1/insulin and activation of Sirt1.
Ang II, angiotensin II; *ATM*, ataxia-telangiectasia mutated; *ATR*, ATM and Rad3 related; *CDK2*, cyclin-dependent kinase 2; *CDK4*, cyclin-dependent kinase 4; *CDK6*, cyclin-dependent kinase 6; *IGF-1*, insulin growth factor 1; *p16*, $p16^{INK4a}$, cyclin-dependent kinase inhibitor 2A; *p21*, $p21^{Cif1/Waf1}$, cyclin-dependent kinase inhibitor 1; *p53*, tumor protein p53; *Rb*, retinoblastoma; *ROS*, reactive oxygen species; *SASP*, senescence-associated secretory phenotype; *Sirt1*, sirtuin 1.

individuals showed less proliferation capacity in culture than podocytes isolated from patients with kidney disease, suggesting podocyte cell senescence as the key mechanism for podocyte loss in healthy individuals [79].

As terminally differentiated, highly specialized epithelial cells, podocytes do not proliferate under physiological conditions, and rarely during an injury. The quiescent state of a podocyte is maintained by expressing several different CDK inhibitors, p21, p27, and p57, with the latter two being only partly detected in other glomerular cells [80]. In addition, attachment to the GBM might contribute to maintaining the differentiated state of podocytes [81]. In vitro, only dedifferentiated podocytes proliferate [82]. In vivo, proliferation of cells in a visceral location, i.e., the usual anatomical site of podocytes, has only been observed in specific pathologies, e.g., in the cellular variant of

segmental glomerulosclerosis, collapsing glomerulopathy, or HIV-associated nephropathy [83]. However, these cells do not express podocyte markers, but rather show proliferation markers and markers of PECs [84]. In fact, in most human diseases or animal models of kidney disease, no proliferation of podocytes is seen [84–86]. Calculations based on the observed rate of podocyte number decline in CKD patients suggest that without a regenerative mechanism, complete podocyte depletion with resulting end-stage kidney disease would occur in all humans within ~ 20 years [77]. Since this is not the case, replacement of podocytes in vivo must occur.

Indeed, there is evidence for a reversal of established kidney disease or halting the progression of aging-related kidney alterations without podocyte proliferation. For example, reversal of diabetic nephropathy by normalizing glycemia was found in humans 10 years after pancreatic transplantation and after leptin supplementation of leptin-deficient diabetic mice [87, 88]. In aging rats, halting or even reversal of glomerular injury was achieved by inhibitors of endothelin-1 (darusentan) and ACE (perindopril, lisinopril) or angiotensin receptor blockers [89–92].

To date, three different sources of podocyte replacement have been postulated: pluripotent stem cells from the bone marrow, PECs, and cells of renin lineage [93]. Several studies suggested the potential of bone marrow pluripotent stem cells to replace podocytes using Y-chromosome detection in female kidneys transplanted to a male recipient. Although the Y-chromosome was detected in podocytes in such transplanted female kidneys in both humans and mice, the percentage of Y-positive podocytes was < 1% [84, 94]. Whether the low number represents low sensitivity of the assay, low turnover of podocytes, or limited capacity of bone marrow to replace podocytes is currently unknown. A study using lineage tracing of de novo podocytes in mice reported no such podocytes following bone marrow transplantation in aged mice [93].

The PECs at the urinary pole are considered multipotent progenitors, since they express markers of stem cells CD24 and CD133, allowing them to transdifferentiate to other cell types [95]. Experiments using lineage tracing of PECs in mice showed that after podocyte injury by Adriamycin, or antipodocyte antibody, PEC lineage cells expressing markers of podocytes de novo were found on the glomerular tufts [96, 97]. A role of PECs in podocyte replacement during aging has been found in some, but not all, lineage tracing studies [93, 98].

Cells of renin lineage (CoRL) are renin secreting, modified vascular smooth muscle cells located at the juxtaglomerular apparatus [99]. They can serve as progenitors for several different cells, such as mesangial cells, smooth muscle cells, myofibroblast-like cells, and also glomerular epithelial cells [100–102]. Lineage tracing of CoRL in mice confirmed their migration onto the glomerular tuft and de novo expression of podocyte markers in these cells after podocyte depletion using antipodocyte antibody or subtotal nephrectomy [103, 104]. CoRL also may differentiate into PECs. Glomeruli containing these cells showed less scarring. In aged mice, CoRL migrated to glomeruli, providing a possible compensatory mechanism to age-related podocyte loss [105].

Unlike podocytes, PECs are known to proliferate in response to injury for either self-renewal or as a progenitor for other cell types. A comprehensive description of PECs alterations in mice during aging showed that the total number of PECs in young vs old kidneys was only different in glomeruli in the outer cortex [106]. However, the density of PECs along Bowmańs capsule was decreased in aged kidneys in both cortical and juxtamedullary glomeruli. In addition, in aging mice, increased collagen IV deposition in Bowmańs capsule was linked to increased expression of CD44, a marker of activated PECs, and an increase in some epithelial-to-mesenchymal transition markers (vimentin, α-SMA). Interestingly, markers of cell senescence, such as p21 or mTOR, were not detected in PECs, indicating an absence of cell senescence in murine PECs.

Cell senescence of tubular epithelial cells

In both aging and injury, tubular epithelial cells (TECs) are most likely to become senescent and are the most abundant senescent cells in the kidney. The underlying factors are (i) the tubular epithelium represents approximately 90% of all renal cells, (ii) a high number of mitochondria per cell, and (iii) proliferative response to external stimuli. Reabsorption and secretion of solutes are energy demanding, requiring a high metabolic rate and a large number of mitochondria, especially in the proximal tubules [107]. Mitochondria are the primary source of reactive oxygen species responsible for oxidative stress-induced damage to macromolecules, such as DNA, lipids, and proteins. During aging, accumulation of such molecules and age-related tissue injury occur [108]. This can be partially attributed to the reduction of mitochondrial turnover in aging [18]. In rats, aging-induced mitochondrial alterations in TECs include swelling and disintegration of cristae, deletions in mtDNA, electron transport system alterations, and altered activity of mitochondrial enzymes [60, 109].

Under normal conditions, the proliferation rate of tubules is minimal, but a self-renewal proliferation of tubules occurs in response to injury [110]. If the injury is too severe or repeated, a maladaptive repair with cell cycle arrest at the G2/M phase occurs in some cells [111]. These cells then produce profibrotic factors and contribute to the development of fibrosis. In aging, the proliferative potential of tubular cells is decreased [112]. At the same time, a cell cycle inducing

factor, cyclin D1, was higher in old mice kidneys vs young [113]. Moreover, there was an inverse correlation between cyclin D1 expression and the proliferative potential of the aged kidney cells. These data suggest an ambivalent role for cyclin D1 in cell cycle regulation. Also, since cyclin D1 mediates cell cycle promotion during the G1/S phase, whether cyclin D1 might be a discriminating factor and/or marker to distinguish injury vs age-related cell senescence remains to be determined. In human kidney biopsies, markers of cell senescence in tubular cells were detected in chronic allograft nephropathy, diabetic nephropathy, minimal change disease, focal segmental glomerulosclerosis, and IgA nephropathy [74, 114–116]. $p16^{INK4a}$ was found in TECs in both aged human and rat kidneys [72, 73]. A comparison of the key characteristics of two essential renal epithelial cell types—podocyte vs proximal tubular cell—is shown in Fig. 21.3.

Senescence in other renal cells

Endothelial cells (ECs) in the kidney include glomerular endothelial cells, peritubular capillaries, and ECs in large and small vessels [117]. In general, the proliferation rate of endothelial cells is higher than that of epithelial cells, mainly promoted by angiogenic factors. Data on cell senescence in specific renal ECs are scarce. In aged rats, the density of both glomerular endothelial cells and peritubular capillaries was decreased [118]. In addition, the proliferation rate in the aged kidneys was decreased in both capillary types. In humans with diabetic kidney disease, there was no difference in $p16^{INK4a}$ staining in glomerular endothelial cells or arterial vessels compared with control biopsies [114]. On the contrary, in streptozotocin-induced diabetic nephropathy in mice, cell senescence was predominantly observed in glomerular endothelial cells [118]. In aged humans, cell senescence by $p16^{INK4a}$ was only detected in a few endothelial cells of arterial vessels and peritubular capillaries in the medulla [72].

Mesangial cells provide support to the glomerular tuft by tethering the GBM and by secretion of ECM [119]. Cell senescence in these cells can be induced by hyperglycemia [120]. As discussed earlier, CoRL can replace mesangial cells after injury. Whether these cells also modify mesangial cell responses to aging is not known.

Molecular signatures of the aging kidney

Early studies in lower organisms showed that a number of different genes can influence lifespan [3]. More recently, high-throughput transcriptomic analyses based on microarrays showed that small differences in a large number of genes (255–985) and not large changes in the expression of a small number of genes are responsible for the

Podocyte

	Healthy	Injured	Senescent
Terminal Differentiation	yes	yes	yes
Cell cycle arrest	G1/S	G1/S	G1/S? G2/M?
Proliferative capacity	-	-	-
Regenerative capacity	External source	External source	External source
Mitochondria/ oxidative stress	low/low	low/low	low/?
DNA damage	low	?	high
Markers of senescence	-	p16	p16

Proximal tubular epithelial cell

	Healthy	Injured	Senescent
Terminal Differentiation	yes	yes/ dedifferentiation	Yes
Cell cycle arrest	G1/S	G2/M	G1/S? G2/M?
Proliferative capacity	low	high	low
Regenerative capacity	high	high	low
Mitochondria/ oxidative stress	high/low	high/high	low/high
DNA damage	low	high	high
Markers of senescence	-	p16, p21, p27 SA-β-GAL	p16, p19, p27 SA-β-GAL

FIG. 21.3 Cell-specific characteristics determine the response to injury and aging.
As an example, two key epithelial cells in the kidney, the podocyte and proximal tubular epithelial cell, show marked differences in their responses to both injury and aging.

aging process [121, 122]. RNAseq analysis of a large number of human kidneys ($n = 563$) and other tissues identified 37 age-related genes in the kidney from which 19 genes were confirmed by subsequent analysis of existing independent web-based gene sets [123]. The majority of the genes correlated with at least one functional or biochemical determinant of aging-associated changes in the kidney. Transcriptomic analysis of isolated podocytes from aged mice revealed a high number of differentially expressed genes (2494) compared with young mice [124]. The top differentially expressed genes from the gene ontology (GO) term "cell senescence" included 20 upregulated genes in aged podocytes, including CD44 and Cebpb, and 15 downregulated genes. In addition, 13 SASP differentially expressed genes (11 upregulated) were identified in the aged podocytes. Transcriptomic analysis of the kidneys from a bowhead whale (*Balaena mysticetus*), the longest living mammal on earth (lifespan ~ 211 years), identified approximately 50 upregulated genes compared with other mammals [125]. Among the identified genes were those associated with DNA repair, tumor suppression, and the mitochondrial stress inhibitor JunB. Whether these are responsible for protection against age-related kidney damage in whales or represent potential targets for intervention in other species remains to be determined. The bowhead whale kidney also shows genome duplication of proliferating cell nuclear antigen (PCNA), a molecule involved in DNA repair, with high PCNA protein expression in the kidneys [126].

Regeneration of aging kidney

Cells within the kidney have variable capacity to regenerate, with decreasing potential with aging. Currently, there is no clear evidence that aging kidneys could be totally reversed to the young kidney phenotype. The balance of senescence and regeneration determines the rate of the aging process. Goals to regenerate and decrease aging-related processes, discussed earlier, are to shift this balance by reducing senescence and amplifying regeneration. The potential regeneration of the aging kidney needs to also be viewed in the context of systemic modifiers of aging. We therefore discuss cellular and systemic bases of renal regeneration in aging (Fig. 21.4 and Table 21.1).

Cellular basis of renal regeneration

Amelioration of cell senescence

Targeting DNA damage

DNA damage occurs continuously, induced by exogenous (radiation, diet, etc.) or endogenous sources (reactive oxygen species, reactive nitrogen species, etc.). DNA damage lesions include base modifications, single-strand breaks (SSBs), double-strand breaks (DSBs), and interstrand cross-links (ICLs). In humans, prominent DNA repair pathways

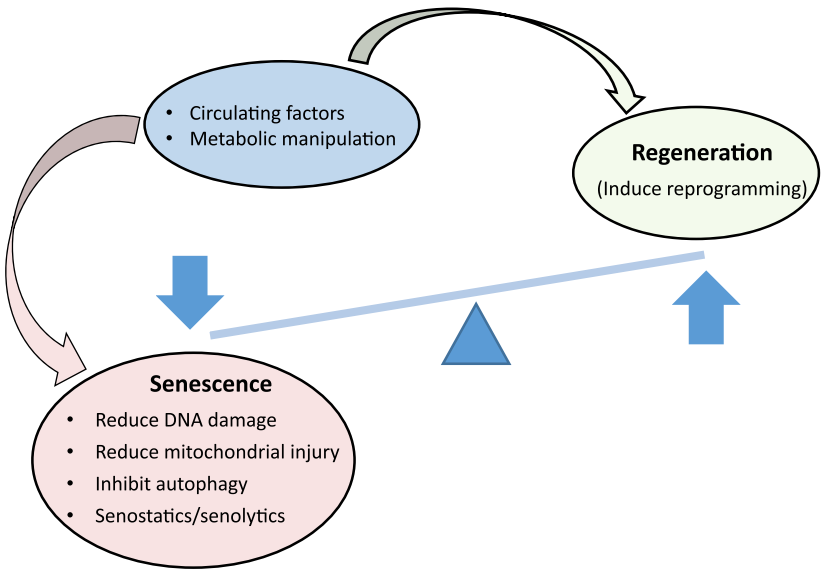

FIG. 21.4 Battle of senescence vs regeneration in the aging kidney.
Strategies to halt renal aging should aim at restoring the balance between the regenerative potential and cell senescence which could be regulated by circulating and metabolic factors.

TABLE 21.1 Studies of renal regeneration in aging.

Treatment	Major mechanism	Model system	Extends life span	Aging kidney effects	References
Targeting DNA damage					
Aspirin	Stimulates NER, senostatic	*C. elegans*/*D. melanogaster*/mouse/rat	Yes	Not studied	[135, 142]
TA-65	Elongates short telomeres	Mouse/rat	Yes	Improves renal function	[138, 139]
Targeting mitochondria					
Nicotinamide mononucleotide	NAD^+ precursor, activates PARP1	Mouse/*C. elegans*	Yes	Protects against AKI in aged mice	[131, 132, 146, 147]
Nicotinamide riboside	NAD^+ precursor, activates PARP1	Mouse/*C. elegans*	Yes	Not studied	[148]
Resveratrol	Activates AMPK/NAD^+	*C. elegans*/*D. melanogaster*/mouse/rat	No	Improves renal function and morphological lesions of aging	[142, 149–151]
Metformin	Activates AMPK, improves metabolism	*C. elegans*/*D. melanogaster*/mouse/rat	Yes	Improves renal function and cell senescence	[140–143]
SS-31	Targets cardiolipin	Mouse	Not studied	Improves glomerulosclerosis and cell senescence	[153]
Targeting autophagy					
Rapamycin/rapamycin analog	Inhibits mTOR activity	*C. elegans*/*D. melanogaster*/mouse/rat	Yes	Improves morphological lesions of aging	[142, 154–157]
Removal of senescent cells					
Dasatinib + quercetin	Senolytic	Mouse	Yes	No	[162]
FOXO4-DRI	Inhibits FOXO4	Mouse	Not studied	Improves renal function and cell senescence	[163]
Fisetin	Senostatic	Mouse	Yes	Not studied	[169]
Replacement/reprogramming of senescent cells					
OSKM	Induces cell reprogramming	Mouse	Yes	Improves morphological lesions and cell senescence	[183]
Circulating factors					
GDF11	Growth factor	Mouse	No	Improves the repair of AKI in aged mice	[189, 190]
Enalapril/losartan	RAAS inhibition	Mouse/rat	Yes	Improves and reverses glomerulosclerosis	[71, 92, 197, 198]
Rhein	Increases Klotho	Mouse	Yes	Improves renal function and morphological lesions of aging	[204]
Metabolic manipulation					
Fasting/ketogenic diets	Caloric restriction	Mouse	Yes	Not studied	[209, 211]

AKI, acute kidney injury; *FOXO4-DRI*, the D-retro inverso (DRI)-isoform of forkhead box O 4; *GDF11*, growth differentiation factor 11; *mTOR*, mammalian target of rapamycin; *NAD*, nicotinamide adenine dinucleotide; *NER*, nucleotide excision repair; *OSKM*, Oct3/4, Sox2, Klf4, and c-Myc; *PARP1*, poly(ADP-ribose) polymerase 1; *RAAS*, renin-angiotensin-aldosterone system; *SS-31*, Szeto-Schiller peptide-31; *TA-65*, telomerase activator-65.

involve the Fanconi anemia (FA) pathway which is specific for repairing ICLs, the base excision repair (BER) mechanism which excises oxidated and alkylated nucleotides, the excision repair (NER) pathway which removes bulky/helix-distorting lesions from DNA, mismatch repair (MMR) which reverses replication errors, and double-strand break repair (DSBR) which is specific for repairing DSBs [127]. Some patients with inherited defects in DNA repair proteins show features of premature or accelerated aging [128]. The capacity to repair DNA damage declines with aging, leading

to genomic instability and cell senescence or cell death [129]. Compared with nuclear DNA, mtDNA mutates at a much faster rate and accumulates more damage in aging because of its close vicinity to a source of ROS [130]. Cellular DNA damage activates the DNA damage sensor poly(ADP-ribose) polymerase 1 (PARP1) to recruit DNA repair proteins and induce depletion of the substrate NAD^+. PARP1 is located in nuclei and mitochondria and is involved in BER, NER, and DSBR. Increased PARP1 activation is detected in aging *C. elegans*, while PARP inhibitor extends *C. elegans* life span accompanied by reduced NAD^+ consumption [131, 132]. Some additional molecules have been suggested to directly stimulate DNA repair. RAD51-stimulatory compound 1 (RS-1) increases DSBR, nicorandil stimulates BER through the APE1 enzyme, and aspirin has been suggested to stimulate NER [133, 134]. Aspirin increases the lifespan of mice, but whether this effect is due to stimulation of DNA repair only, and/or additional mechanism(s), is unknown [135].

Telomeres (TLs) play major roles in stabilizing the genome and modulating DNA repair, and they are usually shortened with aging, as discussed earlier [136]. The maintenance of TLs is ensured by two mechanisms involving telomerase enzyme and alternative lengthening of telomeres. Reactivation of telomerase by gene therapy in old mice delayed aging and significantly extended lifespan without increasing cancer susceptibility [137]. TA-65, a chemical compound extracted from *Astragalus membranaceus*, also elongated short telomeres, increased health span, and alleviated the kidney dysfunction in aging rodents [138, 139]. However, whether these effects translate to humans is questionable, given the very long telomeres in mice vs humans, as discussed earlier.

Targeting mitochondria

Mitochondria produce ATP and generate ROS. As a dynamic organelle, the number of mitochondria is determined by the generation of new mitochondria (biogenesis) and the removal of dysfunctional mitochondria (mitophagy). AMP-activated protein kinase (AMPK) promotes mitochondrial fission and mitophagy. Metformin triggers AMPK activation, facilitates mitochondrial fission and mitophagy, and decreases inflammation when administered starting at middle age [140]. In several animal models, metformin extended life span with reduced cellular senescence in the aging kidney [140–143]. Metformin is now being evaluated in a longevity study in human (https://clinicaltrials.gov/ct2/show/NCT02432287). AICAR (5-aminoimidazole-4-carboxamide-1-β-ribofuranoside), which mimics the action of AMP on AMPK, stimulates mitochondrial biogenesis [144]. In addition, NAD^+ is a central metabolic cofactor that plays a critical role in regulating mitochondrial bioenergetics. NAD^+ is markedly reduced with age [145]. Supplementing NAD^+ intermediates, such as nicotinamide riboside and nicotinamide mononucleotide, improved age-related energy metabolism, physical activity, and insulin sensitivity in mice [146–148]. Resveratrol can also activate AMPK and elevate NAD^+ levels and has been studied as an antiaging compound. Several rodent studies showed that resveratrol improved renal function and reduced inflammation when it was initiated at mid-to-late age [149–151].

Cardiolipin, which is expressed on the inner mitochondrial membrane, is another target for promoting mitochondrial efficiency. It organizes the respiratory complexes into supercomplexes and determines whether cytochrome *c* acts as an electron carrier or peroxidase. Szeto-Schiller peptide-31 (SS-31) binds selectively to cardiolipin, promotes oxidative phosphorylation, and reduces ROS production [152]. In aging mice, SS-31 treatment for 2 months restored mitochondrial cristae structure in podocytes and PECs, with less p16 and SA-β-gal (senescence markers), and reduced glomerulosclerosis [153]. This study indicates that improvement of cellular bioenergetics even at a late stage of aging can delay further renal aging.

Targeting autophagy

Compromised autophagy reduces lifespan and precipitates premature aging in different models, while enhanced autophagy promotes longevity. Rapamycin, the first FDA-approved longevity drug, strongly inhibits mTOR activity. The beneficial effects of rapamycin are most related to mTORC1, which is acutely sensitive to rapamycin, while many side effects of rapamycin are related to the inhibition of mTORC2 after long-term exposure. Administration of rapamycin extended lifespan up to 14% in female and 9% in male mice [154]. Three months of rapamycin treatment increased life expectancy by up to 60% in middle-aged mice [155]. Knockdown of autophagy-related genes abrogated the life-extending effects of rapamycin, indicating autophagy plays a key role in mTOR-mediated life extension [156]. In addition, a rapamycin analog (RAD001) reversed the expression of age-regulated genes in the kidney with a reduction of glomerulosclerosis in aged rats [157].

Removal of senescent cells

Cellular senescence is a main causal factor for aging. Genetically clearing p16Ink4a-positive cells in mice extended median lifespan and attenuated age-related deterioration of several organs, including the kidney [158]. Approaches to therapeutically target cellular senescence include selectively eliminating senescent cells (senolytics) and modulating a proinflammatory senescent secretome (senostatics).

Senescent cells do not divide and show metabolic shifting, epigenetic changes, and resistance to apoptosis. Gene profiling showed that antiapoptotic regulators including the PI3K/Akt pathway and BCL-2 family were increased in senescent cells [159]. Based on this finding, two senolytics were identified. Dasatinib (an FDA-approved tyrosine kinase inhibitor) induced apoptosis of senescent fat cell progenitors, while quercetin (a flavonoid present in many fruits and vegetables) was more effective for senescent endothelial cells [159]. A clinical trial (NCT02848131) testing dasatinib and quercetin as senolytics in diabetic patients with chronic kidney disease is in process [160]. Navitoclax, a BCL-2 family inhibitor, was more effective for human umbilical vein endothelial cells and lung fibroblasts but not for primary preadipocytes [161]. Thus, senescent cells in aging show heterogeneity in responses to various targeting approaches. Optimal strategies to target aging and senescence thus have to consider whole pathways instead of a single gene, and several survival pathways instead of only one universal pathway. Indeed, the combination of dasatinib and quercetin alleviated physical dysfunction and increased posttreatment survival by 36% in radiation-exposed and progeroid mice while reducing mortality hazard to 65% [159, 162]. In aging kidneys, senescence is induced mainly in tubular cells due to increased oxidative and cellular stress. FOXO4 drives nuclear localization of p53 and suppresses the mitochondrial apoptotic pathway. The FOXO4 inhibitor peptide (FOXO4-DRI) reduced SA-β-GAL activity and IL-6 expression in tubules and ameliorated renal dysfunction in aging mice [163]. All these findings suggest that eliminating senescent cells can rejuvenate tissue and organs [72]. However, senescence is important for wound healing, including optimal recovery from renal injury at key time-points [164]. A delicate balance is required to promote adaptive rather than maladaptive repair when using optimal senolytic therapies. Because senescent cells take weeks to form, senolytics can be administered in a "hit-and-run" fashion and potentially still have beneficial effects [165].

SASP is another critical aspect of senescent cells, and senostatics could either modulate the SASP regulatory network in senescent cells or inhibit a specific component of the SASP that exerts deleterious effects [166]. Rapamycin and metformin are effective senostatics in mouse models, but they are not specific and may affect healthy cells [167]. Polyphenols, naturally based compounds, have antioxidant and antiinflammatory properties and could be potential senostatics [168]. Fisetin, a member of the flavonoid family, reduced IL-6, IL-8, MCP-1, and SA-β-Gal activity and prolonged life span in aging mice [169]. Based on the above and additional targets and pathways, multiple early-stage clinical trials centered on modulating senescent cells are underway and early reports are encouraging [160, 170].

Replacement/reprogramming of senescent cells

Stem cells play an important role in the homeostasis, development, and repair of tissues and organs by replacing senescent or damaged cells. However, like other aging somatic cells, stem cells also accumulate cellular damage with time, which can arise from DNA damage, epigenetic changes, and metabolic stress. Age-dependent dysfunction of stem cells includes decreased regenerative and differentiation potential which impairs their ability to repair damaged tissue. In organs with moderate to high cell turnover, such as the gut and bone marrow, aging appears to be determined by senescent stem cells [171]. In contrast, in organs with low cell turnover, such as the kidney and the heart, aging is mostly related to senescent parenchymal cells [172]. However, senescent stem cells from bone marrow contribute to renal aging. Our group studied differences of aging vs young bone marrow–derived stem cells and their contribution to renal aging. Aging mice receiving young bone marrow had minimal progression of proteinuria, and much less proteinuria than the mice receiving old bone marrow. By double staining, using the Y chromosome as a marker in these male-to-female bone marrow transplant mice, we determined that bone marrow cells did not directly replace parenchymal cells. Rather, our results support the theory that bone marrow–derived cells have paracrine effects on renal parenchymal cells, including effects mediated by Klotho [173]. Numerous studies have characterized a role of stem cell-derived extracellular vesicles (SCEVs) in age-associated conditions, or in a tissue-specific context, SCEVs contain mRNAs, miRNAs, proteins, lipids, transcription factors, cytokines, and growth factors, which affect target cells through receptor-ligand mediated signaling or direct membrane fusion and endocytosis [174]. SCEV-mediated cell communication has been implicated in numerous aging processes, including tissue regeneration, reprogramming, differentiation, and telomere function [175, 176]. For instance, miR-183 level in bone marrow–derived SCEVs was elevated in aged mice, which suppressed stem cell function and induced cellular senescence [177]. However, little is currently known regarding how populations of SCEVs can regulate whole-body processes in aging.

Induced pluripotent stem cells (iPSCs) closely resemble embryonic stem cells and can be induced by four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc (OSKM), named Yamanaka factors, from somatic cells through the global remodeling of epigenetic marks [178]. iPSCs induction is a multiphased process including initiation, maturation, and stabilization [179]. Some senescent phenotypes of aging were rejuvenated by the reprogramming process when studied in vitro [180, 181]. The transient expression of these nuclear reprogramming factors ameliorated cellular aging and restored a youthful regenerative response in human endothelial cells and fibroblasts, through resetting of the epigenetic

clock and reduction of the inflammatory profile [182]. In a mouse model of premature aging, partial reprogramming by short-term OSKM extended the lifespan and reduced renal tubular atrophy by reducing DNA damage and mitochondrial ROS and by restoring H3K9me3 [183]. This reprogramming also increased resistance to metabolic disease and muscle injury in aging mice [183]. These epigenetic and transcriptional changes occurred before cellular identity was affected by epigenetic reprogramming. These data support the importance of epigenetic regulation of mammalian aging. Whether the cellular reprogramming is uncoupled from the dedifferentiation process is unknown [184].

Systemic basis of renal regeneration

Circulating factors

Heterochronic parabiosis experiments, a technique that joins the circulatory system of an old mouse to a young mouse, showed reversal of the aging process in different organs, indicating the contribution of some circulating factors to the regeneration [185]. Old kidneys from young-old parabionts showed reduced apoptosis, senescent marker, and inflammation and increased autophagy after 5 weeks of shared circulation compared with old-old parabionts [186]. In another mouse parabiosis ischemia-reperfusion AKI model, adding young circulating factors to old mice for 3 weeks prior to AKI reduced tubular injury and improved outcome in old mice compared with old-old parabionts [187]. This could be contributed to by pro-rejuvenation factors contained in young blood or by dilution and/or inhibition of proaging factors in aged blood. However, identifying potential rejuvenation factors in young blood has been difficult. Injection of growth differentiation factor 11 (GDF11) alone had similar antiaging effects as young blood on the hearts of aged mice, but did not extend the life span [188, 189]. A GDF11 supplement also increased the proliferation of proximal TECs after ischemic injury and improved functional recovery and survival in aging mice [190]. However, other researchers showed complicated results and cautioned that GDF11 also has the potential to initiate or accelerate cancer [191]. In addition, other factors such as osteocalcin, oxytocin, and TIMP2 have been identified as potential critical factors in young plasma for regeneration of brain, skeletal muscle, and other organs. Their potential effects on kidney aging are not known [192–194].

Regarding established pharmaceutical approaches, there is a paucity of data on the effects on aging. Such studies are inherently difficult and time consuming to perform. Murine studies showed that inhibition of RAAS reduced cardiovascular aging and increased expression of prosurvival genes including sirtuin 3 in the kidney [71]. Moreover, losartan or spironolactone decreased the expression of $p16^{INK4a}$ in kidneys of hypertensive rats and reduced oxidative stress in the mitochondria, indicating that RAAS activation may be a druggable mechanism contributing to aging [195, 196]. Enalapril or losartan substantially prolonged life span with improved renal function and morphology in aging rats [197, 198]. Our group found that losartan not only slowed the progression of glomerulosclerosis but also induced regression in aging rats [92]. Long-term angiotensin II infusion downregulated expression of renal Klotho, a key aging-related molecule, suggesting that Klotho may in part mediate mechanisms of RAAS inhibition to improve aging [199]. In the aged monkey, Klotho was epigenetically modified by methylation, with resulting less Klotho expression in kidney [200, 201]. Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is a lipophilic anthraquinone compound isolated from rhubarb used to treat various disorders including diabetic nephropathy [202]. Rhein corrected DNA methyltransferase aberrations and reversed DNA hypermethylation-associated Klotho suppression [203]. Aged mice treated with rhein had prolonged median survival time by approximately 25% with less renal fibrosis [204]. Thus, Klotho has multiple complex interactions with various aging-related mechanisms.

Metabolic manipulation

Caloric restriction extends lifespans across different species resulting in reduced glomerulosclerosis, tubular atrophy, and interstitial fibrosis [60, 205, 206]. Even short-term caloric restriction (5 days to 12 weeks) improved function in multiple tissues of aging mice [207, 208]. The periodic fasting-mimicking diet (FMD) repeats cycles of very low caloric intake (4 days) twice per month with ad libitum feeding in between. FMD increased median lifespan and decreased the incidence of cancer and inflammatory disease in aging mice [209]. The ketogenic diet reduces carbohydrate consumption which mimics the metabolic changes in fasting mice [210]. The ketogenic diet increased median lifespan and improved motor function in aging mice [211]. Some features, such as kidney weight and lymphoid to myeloid ratio, were similar or better posttreatment compared with pretreatment, suggesting that this diet change not only delays but may also reverse aging [209]. In addition, diets with reduced fructose content might promote healthier kidney aging, and specific nutritional fats also showed beneficial effects [205, 212]. While caloric restriction modulates several nutrient-sensing pathways (AMPK, IGF-1/Insulin, mTOR, etc., discussed earlier), it also affects epigenetic landscapes, such as DNA methylation, histone posttranslational modifications, and age-related loss of heterochromatin [213–215].

Summary

Renal aging is a multifactorial process influenced by both extra-renal (circulating) and intra-renal factors. The kidney contains many different cell types, including highly specialized epithelial cells, contributing to glomerular filtration (e.g., podocytes) and solute reabsorption (TECs). These cells respond differently to injury and aging and have different regenerative potential. Such differences are determined by their differentiation, overall morphology, and organelle composition, e.g., abundance of mitochondria, and overall proliferative potential. Oxidative stress is more likely to contribute to tubular cell senescence, related to the high workload and abundant mitochondria in these cells, and antioxidative therapies might be more effective in this kidney segment. On the other hand, in large-scale human population-based studies, antioxidative treatments mostly failed to show health benefits. Whether kidney-specific antioxidant therapies could halt renal aging in humans is currently unknown.

Cell senescence is a key characteristic of aging and also the main focus of strategies to halt aging. However, the identification of senescent cells is not straightforward. Markers of cell senescence can be nonspecific, expressed by nonsenescent cells, and different cells might express varying senescence markers. Therefore, identification of novel, more specific markers of cell senescence is warranted.

One of the strategies to reverse cell senescence would be retaining or regaining the proliferative potential of these cells. While proliferation is crucial for tissue regeneration, uncontrolled proliferation is the hallmark of cancer. Thus, overriding the balance between senescence-driven loss of proliferation and its benefit for tissue regeneration could be detrimental. Because a complete stop of the aging process is implausible, a more realistic goal is to halt or slow its progression. Since aging is a multifactorial process, it is unlikely that therapeutic manipulation of only one pathway will be successful. On the contrary, fine-tuning multiple pathways to slow down the shift to cell senescence while preventing excessive proliferation could be a more useful strategy. Since aging is a long-term process, and some of its signs can be observed early in adulthood, it is also essential to consider the onset and the duration of such treatment.

While experimental animal and cell models are invaluable in biomedical research, the translation of results to humans is often complicated. This might be especially true for aging, not only because of the already mentioned biological differences in lifespan or telomere lengths vs rodents, but also environmental, social, cultural, and other nonbiological parameters typical for humans that affect well-being, health, and aging. In addition, some epigenetic effects, such as microRNAs, or comorbidities, and multipharmacological treatments, show significant differences between humans and other species. Therefore, translation failures of experimental model research to the human condition are likely, if the integrated physiological, pathophysiological, and social contexts of aging are not considered.

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Figs. 21.1, 21.2, and 21.3 are created using BioRender.com.

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CHAPTER

22

Gender-dependent mechanisms of injury and repair

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Our knowledge of the influence of sex hormones on the regeneration of injured renal tissue is extremely limited. In contrast, the effects of sex hormones on the regeneration of injured cardiac, hepatic, neural, and musculoskeletal tissue have been explored in depth. These studies provide far-reaching insights into the role of sex hormones in the regeneration of injured tissue and provide direction for further research exploring the relationship between sex hormones and repair after renal injury.

Sex hormones and mechanisms of renal injury

In numerous experimental models of renal disease, the severity of renal injury is intensified and its progression accelerated in male animals [1–3]. Gender dimorphism in the course of renal disease is replicated by hormonal manipulation, suggesting that the hormonal milieu, rather than genetic differences between the sexes, are primarily responsible for gender dimorphism [1–3]. As shown in Fig. 22.1, sex hormones influence many of the processes known to mediate progressive renal injury, including cell proliferation, mesangial matrix synthesis and degradation, generation of reactive oxygen species, and the expression of proinflammatory and profibrotic cytokines, hormones, and vasoactive agents [1–3]. The progression of chronic kidney disease in humans is arguably also more rapid in men than in women, a finding which parallels observations in experimental animals [1–3].

Cellular metabolism

We have shown that serum-stimulated mesangial cell proliferation is inhibited by physiologic concentrations of estrogen via estrogen receptor (ER)-dependent mechanisms [4]. Estrogen stimulates the proliferation of Syrian hamster renal proximal tubular cells via ER-dependent mechanisms but does not alter the growth of rat or guinea pig proximal tubular cells or Syrian hamster renal medullary cells [5].

The accumulation of glomerular extracellular matrix reflects the balance between matrix synthesis and matrix degradation, both of which are influenced by sex hormones. We demonstrated that estradiol suppresses type I collagen synthesis in mesangial cells by upregulating the mitogen-activated protein kinase/activator protein 1 signal transduction pathway [6, 7]. We have also shown that mesangial cell type IV collagen synthesis is suppressed by estradiol, mediated by complex interactions with transforming growth factor-β1 (TGF-β1) [8, 9]. In addition, we and others have shown that estradiol enhances matrix degradation by upregulating metalloproteinase 2 and metalloproteinase 9 activity [10, 11]. The effects of estradiol on metalloproteinase 2 protein levels and enzyme activity are mediated by the upregulation of the mitogen-activated protein kinase/activator protein 2 pathway [10]. By suppressing the synthesis of mesangial matrix components as well as increasing the rate of matrix degradation, estrogen reduces the accumulation of glomerular extracellular matrix and attenuates the development of glomerulosclerosis following renal injury.

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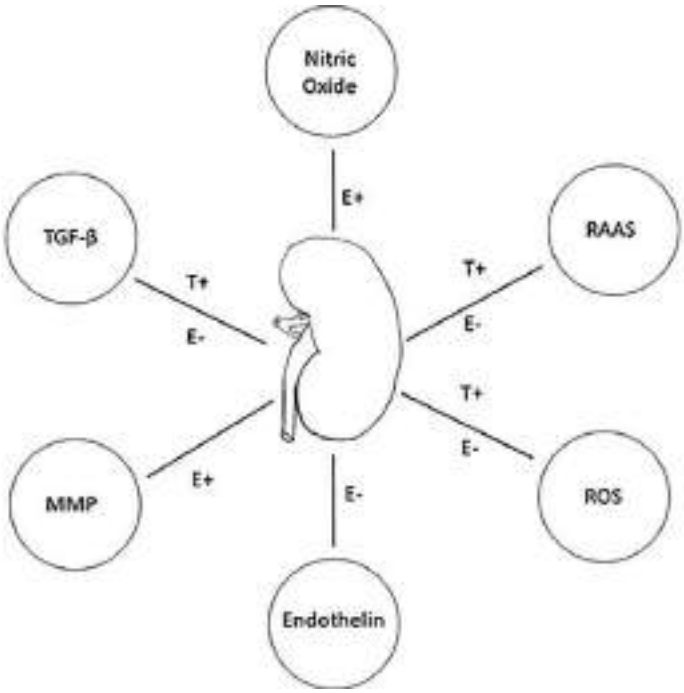

FIG. 22.1 Mechanisms by which sex hormones influence the development and progression of renal injury. Abbreviations: *E +*, estrogen stimulates the pathway; *E −*, estrogen inhibits the pathway; *MMP*, matrix metalloproteinases; *ROS*, reactive oxygen species; *RAAS*, renin, angiotensin, aldosterone system; *TGF*, transforming growth factor; T+, testosterone stimulates the pathway.

Enhanced generation of reactive oxygen species plays a critical role in promoting renal injury. We and others have shown that, in supraphysiologic concentrations, estradiol is a potent antioxidant [12]. Estradiol also upregulates numerous antioxidant enzymes and protects against renal damage mediated by oxidative stress in experimental models of renal injury [12]. In these models, estrogen reduces the generation of superoxide anion, the major reactive oxygen species produced by the kidney, by attenuating upregulation of nicotinamide adenosine dinucleotide phosphate oxidase activity [12]. Estrogen also increases the expression of glutathione and other antioxidant enzymes including superoxide dismutase. In contrast, testosterone increases oxidative stress after renal injury by increasing the generation of reactive oxygen species and downregulating antioxidant enzymes [12].

Cytokines, growth factors, and vasoactive agents

TGF-β1 plays a key role in promoting progressive renal injury. In numerous animal models of renal disease, administration of estrogen decreases, whereas administration of testosterone increases renal expression of TGF-β1 [13]. Consistent with these experimental observations, circulating TGF-β1 levels are lower in women than in men. [14] Some of the effects of estrogen on TGF-β1 expression may be indirect insofar as estrogen suppresses, and testosterone upregulates, the synthesis and/or peripheral activity of angiotensin II and endothelin-1, both potent inducers of TGF-β1 [15].

Estrogen interferes with the actions of TGF-β at multiple points in the TGF-β signal transduction cascade. Cooperation between Smad proteins and the transcription factor specificity protein 1 is an essential element mediating interactions between TGF-β signal transduction pathways and the transcriptional machinery responsible for the actions of TGF-β [8]. We have shown that estradiol interferes with TGF-β1-induced activation of protein kinase CK2, a ubiquitous serine/threonine protein kinase involved in signal transduction and transcriptional regulation. Protein kinase CK2 mediates TGF-β1-simulated type IV collagen gene transcription by increasing the quantity of free specificity protein 1 available to transactivate the collagen IV promoter. By inhibiting protein kinase CK2, estradiol reverses TGF-β1-stimulated collagen IV synthesis [8].

We also demonstrated that TGF-β1 induces mesangial cell apoptosis via upregulation of protein kinase CK2 activity, which in turn leads to phosphorylation of p53 and initiation of an apoptotic cascade [16]. Again, we showed that estradiol reverses this process by preventing TGF-β-induced activation of protein kinase CK2. Alb/TGF-β1 mice overexpress TGF-β1 and develop proteinuria and progressive renal injury. We have demonstrated that estradiol reverses the injurious effects of TGF-β1 and ameliorates renal injury in this model by preventing protein kinase CK2 activation [17].

In transfected human embryonic kidney cells exposed to estrogen, ERα forms a ternary complex with Smad2/Smad3 and the ubiquitin ligase Smurf [18]. Formation of this complex enhances ubiquitination and degradation of the Smad proteins. These interactions inhibit the TGF-β1 signaling cascade and explain the finding that estrogen decreases the expression of total and phosphorylated Smad2/3 proteins in kidneys harvested from diabetic rats [19]. In addition, estradiol reverses the increased expression of TGF-β1 receptors types I and II in these animals [19].

Nitric oxide (NO) is an important modulator of renal injury [3]. We have shown that the expression of nitric oxide synthase (NOS) in the renal medulla of female rats is greater than that in male rats [20]. In general, upregulation of endothelial NOS (eNOS) ameliorates renal injury, whereas upregulation of inducible NOS (iNOS) promotes renal injury. The promoter region of the eNOS gene contains an estrogen response element. Physiologic concentrations of estrogen increase eNOS messenger ribonucleic acid and protein levels in cultured glomerular and vascular endothelial cells via ERα-dependent mechanisms [21]. Intact female rats have higher eNOS levels than males; a difference that is reversed by ovariectomy [15]. Release of intracellular calcium in response to estrogen/G-protein-coupled estrogen receptor interactions enhances local production of prostaglandin E_2 and prostacyclin which in turn contribute to eNOS activation. Conversely, estrogen attenuates upregulation of iNOS in response to inflammatory stimuli, which plays a protective role in several experimental models of renal injury [15].

Endothelin levels are upregulated in renal disease and play a key role in promoting progressive renal injury. Endothelin is a potent vasoconstrictor that increases blood pressure, stimulates TGF-β synthesis, and promotes oxidative stress. Estrogen inhibits endothelin synthesis and dampens its vasoconstrictor effects, whereas the opposite is true for testosterone [3]. Estrogen may also indirectly reduce endothelin levels by suppressing the synthesis of AII, a potent inducer of endothelin [3]. Endothelin levels are lower in men than in women and lower in premenopausal than in postmenopausal women [22]. Endothelin levels decrease in male-to-female transsexuals treated with estradiol and a progestational agent, whereas endothelin levels increase in female-to-male transsexuals treated with testosterone [22].

Cellular apoptosis

We have shown that estradiol inhibits TGF-β-stimulated apoptosis of cultured mesangial cells by inhibiting phosphorylation of p53 by protein kinase CK2 [16]. Estrogen also protects cultured podocytes from apoptosis induced by testosterone, TGF-β, tumor necrosis factor (TNF)-α, and puromycin aminonucleoside [23, 24]. These protective effects have been attributed to reduced generation of reactive oxygen species, stabilization of mitochondrial membrane potential, and activation of cytoprotective extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3 kinase (PI3K)/Akt signal transduction pathways [23, 24]. In contrast, testosterone induces cellular apoptosis via androgen receptor-dependent mechanisms independent of TGF-β [23]. Testosterone promotes apoptosis of human proximal tubule cells by activating the c Jun amino terminal kinase signal transduction pathway, which in turn upregulates Fas/Fas ligand and activates caspase-dependent apoptotic pathways [25, 26].

Renin/angiotensin/aldosterone system

Estrogen upregulates the expression of angiotensinogen but downregulates the expression of renin, angiotensin-converting enzyme, and angiotensin II [3, 21]. Estrogen increases the expression of angiotensin type 2 receptors in the renal medulla but decreases the expression of angiotensin type 1 receptors in the kidney and in vascular smooth muscle [3, 21, 27]. The estrogen-induced increase in angiotensin type 2 receptor density stimulates bradykinin synthesis, leading to increased nitric oxide and prostaglandin E_2 synthesis [3, 21, 27]. The decrease in angiotensin type 1 receptor density antagonizes sodium retention and renal vasoconstriction [3, 21].

In contrast, testosterone activates the renin/angiotensin/aldosterone system (RAAS) [28, 29]. Dihydrotestosterone increases angiotensin type 1 receptor expression in cultured mesangial cells [28, 29]. Angiotensinogen messenger ribonucleic acid levels are higher in male rats than in female rats; a difference that is reversed by orchiectomy and restored after testosterone replacement [28, 29]. Prorenin and renin levels are higher and plasma renin activity is greater in men than in women [28, 29]. By activating RAAS, testosterone promotes oxidative stress and increases superoxide production, which in turn quenches nitric oxide and decreases vasodilatory responses [28, 29]. Angiotensin II infusion elicits a hemodynamic response that promotes glomerular capillary stress in men but not in women [30]. Clearly, the effects of sex hormones on RAAS contribute to gender dimorphism in the development and progression of renal injury.

Renal hemodynamics

Despite having a similar corrected glomerular filtration rate (GFR), Munger and Baylis [31] found that renal vascular resistance was higher in female rats compared with male rats [31]. They postulated that this difference might protect females from hyperfiltration-induced glomerular injury by attenuating increases in glomerular capillary pressure seen in many forms of renal disease. In addition, the glomerular hemodynamic responses to angiotensin II in humans also differ between men and women [30]. Healthy young adult men respond to an infusion of angiotensin II by maintaining their GFR at the expense of an increased filtration fraction, suggesting an increase in glomerular capillary pressure [30]. In contrast, in response to angiotensin infusion, women show a decrease in both GFR and effective renal blood flow with unchanged filtration fraction, suggesting that there has been no change in glomerular capillary pressure [30]. This dissimilar response to angiotensin II may protect women from hyperfiltration-induced renal injury by attenuating increases in glomerular capillary pressure and reducing glomerular hemodynamic stress associated with progressive kidney disease.

Sex hormones and mechanisms of repair and regeneration after injury

Kidney

Animal models have consistently demonstrated that male sex is associated with more severe functional renal impairment and more histologic damage after ischemia–reperfusion (I/R) injury [32–44]. Ovariectomy or testosterone administration exerts deleterious effects, whereas castration or administration of exogenous estrogen is renoprotective in this model [45, 46]. Sexual dimorphism in ischemic AKI may be mediated by effects of sex hormones on cellular processes instrumental in the pathogenesis of AKI. Sex-related differences in the generation of NO and in the synthesis and vascular response to endothelin, angiotensin II, and numerous other vasoactive and inflammatory agents have been demonstrated in experimental models and in humans. In response to I/R, Na^+-K^+ ATPase enzyme activity is greater in females than in males and is associated with reduced transcellular translocation [33]. Female animals subjected to I/R maintain a renoprotective profile compared with their male counterparts with respect to proteases, caspases, antioxidant enzymes, heat shock protein 72 and cytokines, and signaling pathways that mediate apoptosis and proinflammatory responses [32–44].

Despite the fact that most investigators emphasize the protective role of estrogen in mediating sexual dimorphism in I/R models, Park et al. [40] suggest that the increased susceptibility of males to I/R injury is primarily due to the presence of testosterone rather than the absence of estrogen. In an attempt to elucidate the mechanisms responsible for testosterone's deleterious effects, the investigators found that testosterone attenuated increases in the ratio of ERK to JNK phosphorylation, NOS activity, and Akt phosphorylation associated with renal ischemia, leading to a greater inflammatory response and increased kidney injury. Of note, these actions were independent of the androgen receptor (AR).

Although the KDIGO Clinical Practice Guideline for Acute Kidney Injury concludes that female sex is a risk factor for most forms of AKI in humans, recent data suggest that female sex may actually be renoprotective in this disorder [45, 47, 48]. In a meta-analysis of 14 studies which reported sex-stratified data in over 220 million unselected hospitalized patients adjusted for factors known to influence the development of AKI, we found that men were 1.57 times more likely than women to develop AKI [47]. However, this analysis was limited by heterogeneity in patient populations and in outcome measures and cannot be considered conclusive.

While female sex confers tolerance to renal ischemic injury in animal models, clinical evidence for sexual dimorphism in recovery from AKI is limited. In a study that analyzed the US Veterans Health Administration database, women (albeit representing only approximately 5% of the study population) were at decreased risk of developing CKD after an AKI episode than were men [49]. In contrast, an analysis of the AKI cohort of the largest insurance claims database in the country suggested that women had a lower likelihood of recovery from dialysis-dependent AKI [50]. These findings, however, were likely confounded by differences in the etiology and severity of AKI as well as differences in the therapeutic approach to management of men versus women. For example, initiation of dialysis for AKI occurs later in women as compared with men. These mixed results notwithstanding, evidence from animal studies suggests that females have a better ability to recover from AKI than males.

Cyclic increases in proximal tubular cell turnover in ovulating women may confer enhanced capacity for tubular repair and regeneration. Seppi et al. [51] showed that the temporal pattern of increased urinary excretion of a marker of proximal tubule cell death correlated with the phases of the menstrual cycle in ovulating women. The investigators suggested that the decrease in estrogen levels, which occurs after ovulation and before the onset of menses, leads to proximal tubular cell death and desquamation and conversely, that preovulatory and mid luteal surges in estrogen

levels inhibit proximal tubular cell apoptosis and stimulate cellular proliferation and regeneration. The investigators went on to suggest that this enhanced cellular turnover in ovulating women increases the resistance of tubular cells to injury and enhances their reparative and regenerative capacity.

Few studies have attempted to separate the effects of sex hormones on renal regeneration after established kidney injury from the effects of these hormones on mitigating the original insult. Wu et al. [52] found that the administration of estrogen to adult male rats 2 h after I/R reduced the severity of renal injury, attenuated cellular apoptosis, and accelerated proximal tubule regeneration. Estrogen-treated animals showed a greater tubular proliferative response and upregulation of markers of tubular regeneration. There was evidence of both increased dedifferentiation and redifferentiation of proximal tubule cells, associated with increased expression of vimentin, a marker for epithelial mesenchymal transition, and E-cadherin, an epithelial cell marker. TNF-α and ICAM-1 levels were lower and IL-10 levels were higher in estrogen-treated animals, reflecting reduced inflammation and enhanced endothelial cell survival. The investigators concluded that estrogen accelerates proximal tubule cell regeneration post ischemic injury.

Although Lima-Posada et al. [53] found that the severity of renal injury 24 h after I/R was similar in male and female rats, female showed less evidence of oxidative stress associated with increased renal glutathione levels. Furthermore, the authors demonstrated clear sexual dimorphism in the transition from AKI to CKD. After 4 months, CKD developed only in male and ovariectomized female rats, but not in intact females. Females, but not males, showed upregulated eNOS, hypoxia-inducible factor-1α, and vascular endothelial growth factor (VEGF) expression. The authors suggested that females were protected from AKI to CKD transition post ischemic injury by reduced oxidative stress and inflammation and less chronic hypoxic renal injury; the latter is the result of attenuated vascular rarefaction due to upregulation of hypoxia-inducible factor-1α and VEGF expression.

Adding further complexity in our understanding of sexual dimorphism in I/R injury, Robert et al. [54] found a decline in testosterone plasma levels and a large increase in estradiol plasma levels after I/R, likely related to upregulation of aromatase activity. After 5 days of reperfusion, these investigators found that male rats exhibited more severe tubular injury as compared with females. Proximal tubule regeneration, evidenced by increased cellular proliferation and epithelial mesenchymal transition, was also more pronounced in male rats than in female rats. However, these results may merely reflect the greatest severity of renal injury seen in the males rather than an enhanced capacity for regeneration and repair.

The unilateral nephrectomy model in the rat is useful to examine sex differences in compensatory regeneration of kidney tissue after nephron loss. After unilateral nephrectomy, male rats and testosterone-treated ovariectomized female rats show greater compensatory renal growth and glomerular hypertrophy than intact female rats but suffer more glomerular and tubular damage [54–57]. Compensatory renal growth in adult males is achieved primarily via hypertrophy with minimal hyperplasia and is mediated by growth hormone, independent of IGF-1 [57]. In contrast, compensatory remnant kidney growth in female and juvenile male rats is characterized by a significant hyperplastic component which is mediated by IGF-1, independent of growth hormone [56]. Glomerular AT1 receptor expression increases after unilateral nephrectomy only in adult male rats and is growth hormone-dependent [55]. The authors suggested that increased AT1 receptor expression may mediate early remnant kidney growth in males and promote renal damage [55].

Ischemic preconditioning (IPC) refers to a protective strategy involving delivery of submaximal ischemic insults to target tissues to protect against subsequent I/R injury [58]. IPC has been studied extensively in experimental models of cardiac ischemia where female animals benefit disproportionately as compared with males [58]. The evidence for sexual dimorphism in the protective effects of IPC on renal I/R injury is not as strong as for cardioprotection. Several rodent studies, attempting to reproduce the I/R injury seen in kidneys harvested for transplantation, demonstrated greater protection from I/R after IPC in female animals compared with male animals. This protection was attenuated in females that had undergone ovariectomy, suggesting a role for estrogen [59, 60]. In contrast, recent meta-analysis of IPC was unable to perform subgroup analysis based on sex due to the scarcity of studies comparing males with females [61]. Although some evidence suggests increased protection from I/R after IPC in females, the data are by no means conclusive or robust.

Cardiovascular system

Bone marrow–derived endothelial progenitor cells (EPCs) are pluripotent stem cells capable of differentiating into endothelial cells. These cells express both estrogen receptor isoforms, ERα and ERβ, irrespective of whether they originate in male or female animals [62–64]. EPCs are recruited and mobilized from the bone marrow into the circulation and then to sites of ischemic tissue injury in response to the release of chemotactic cytokines. Circulating EPCs home to sites of tissue injury to repair endothelial damage, promote revascularization, and preserve injured myocardial tissue [65].

Physiologic concentrations of estrogen increase proliferation and clonogenic expansion of EPCs via ER-dependent mechanisms [62–72]. Estrogen also increases EPC migratory and adhesive capacity and promotes mobilization and incorporation into areas of tissue injury to enhance vascular lumen formation and neovascularization [62–72]. Circulating EPCs are increased in number in females of reproductive age compared with males or postmenopausal females [62]. Mobilization of these cells from the bone marrow into the circulation correlates with estrogen levels during the menstrual cycle [62]. The increase in circulating EPCs during the ovulatory phase has been attributed to ERβ-mediated upregulation of eNOS and matrix metalloproteinase (MMP)-9 activity [63]. MMP-9 activation releases soluble kit ligand which facilitates transformation of EPCs from the quiescent to the proliferative state.

It has also been suggested that reduced apoptosis contributes to the estrogen-associated increase in the number of circulating EPCs via ERα- and caspase-8-dependent pathways [73, 74]. Estrogen has also been shown to promote EPC survival and delay the onset of EPC senescence by activating telomerase activity via increased expression of the enzyme's catalytic subunit, telomerase reverse transcriptase, via PI3K/Akt signaling [74–77].

Studies exploring the effects of sex hormones on regeneration of cardiac tissue after experimental ischemia/reperfusion (I/R) injury or myocardial infarction show a cardioprotective effect of estrogen that is consistent between the sexes and across species. Estrogen preserves the integrity of cardiac tissue and enhances functional recovery after ischemic injury. As shown in Fig. 22.2, several distinct mechanisms have been identified which involve effects of sex hormones on endothelial progenitor cells, cardiomyocytes, and cardiac fibroblasts.

In experimental cardiac I/R injury, estrogen augments EPC recruitment and mobilization and promotes homing to sites of cardiac injury [65]. These actions enhance incorporation of EPCs into vascular structures to promote neovascularization and tissue repair [65]. In experimental models of myocardial infarction, ERα KO mice showed impaired EPC mobilization and homing to injured myocardium and reduced VEGF levels compared with WT mice, resulting in reduced periinfarction border zone capillary density and worse left ventricular function [72]. Although neovascularization is also reduced in ERβ KO mice after cardiac ischemia, the magnitude of the effect is markedly diminished, suggesting that the effects of estrogen on EPCs, and ultimately on cardio-protection from ischemic injury, are primarily mediated by ERα.

Yuan et al. [68] studied ovariectomized mice subjected to acute myocardial infarction and found that bone marrow–derived EPCs preconditioned by estrogen exposure showed enhanced mobilization, homing to infarcted myocardium and incorporation into sites of neovascularization. Treated mice showed reduced left ventricular scarring and increased left ventricular function. The effects were mediated by ERα via upregulation of stromal cell-derived factor-1α (SDF-1α) and its receptor, CX chemokine receptor-4 (CXCR4) [78]. SDF-1α is a chemokine secreted by CD34 + hematopoietic progenitor cells, which plays a role in mobilizing and directing bone marrow stem cells. In fact, local administration of

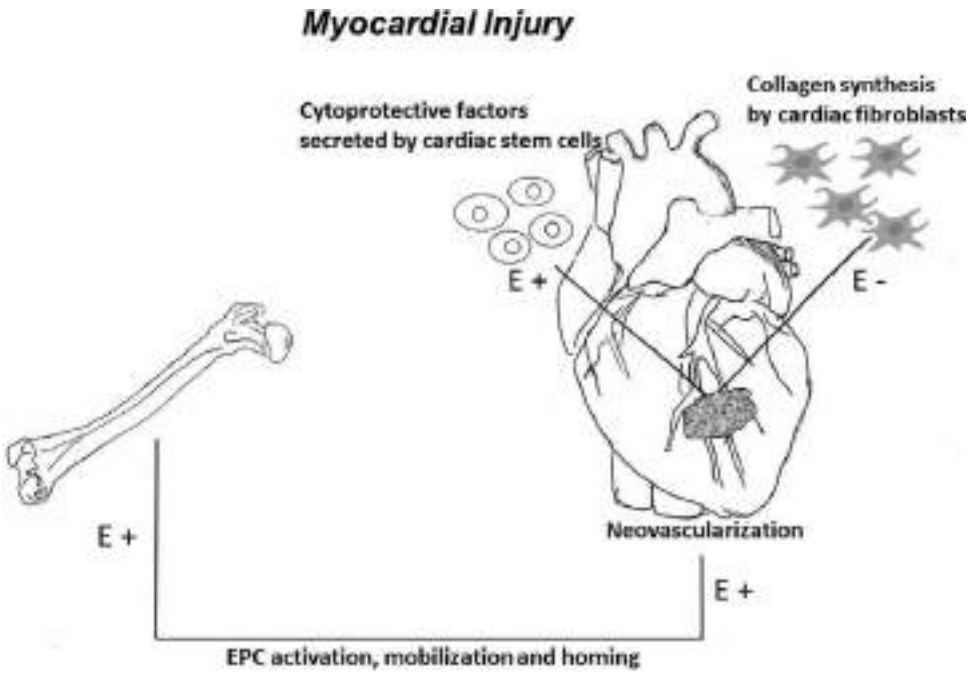

FIG. 22.2 Mechanisms by which estrogen enhances repair and regeneration after ischemic cardiac injury. Abbreviations: *E +*, estrogen stimulates the pathway; *E −*, estrogen inhibits the pathway; *EPC*, endothelial progenitor cells.

SDF-1α after myocardial infarction promotes myocardial neovascularization. An important role for CXCR4 in mediating estrogen's effects was suggested by Li et al. [79] who found that estrogen upregulates EPC CXCR4 expression and that estrogen-induced enhancement of EPC migratory activity was blocked by an inhibitor of CXCR4.

A significant role for eNOS in mediating the cardio-protective effects of estrogen after ischemia was suggested by Iwakura et al. [71]. They showed that the protective effects of estrogen postmyocardial infarction were abolished in eNOS KO mice. The ability of estrogen to preserve the integrity of ischemic cardiac tissue was mediated by enhanced mobilization and incorporation of EPCs into sites of neovascularization which in turn was dependent on eNOS-induced upregulation of MMP-9 expression in the bone marrow.

Estrogen also regulates the activity of mature endothelial cells. The ability of estrogen to increase VEGF secretion and stimulate migration and proliferation of mature endothelial cells is dependent, in part, on its effects on eNOS activity and VEGFR2 [76]. In addition, estrogen exerts direct cardioprotective and regenerative effects. In isolated cardiac myocytes, estrogen partially restores mitochondrial activity after I/R [80]. Estrogen also improves cardiac fibroblast-mediated cardiac remodeling after ischemic injury, resulting in reduced left ventricular scar formation and improved left ventricular function [81]. These effects are, in part, mediated by direct effects of estrogen on cardiac fibroblasts which express ER. Estrogen inhibits cellular proliferation, AT-1 receptor expression, and collagen synthesis in cardiac fibroblasts harvested from both male and female animals. Estrogen also suppresses RAAS gene expression and inhibits RAAS signal transduction in these cells. Suppression of cardiac fibroblast proliferation is mediated by both ERα and ERβ via p42/44 MAPK activation and suppression of p38 MAPK. Other effects of estrogen on cardiac fibroblasts may be mediated by nongenomic activation of large conductance calcium-activated potassium channels.

It has also been suggested that paracrine release of cytoprotective factors by cardiac stem cells mediates estrogeńs cardioprotective effects in models of cardiac ischemia [82–84]. Wang et al. [85] examined mesenchymal stem cell paracrine function and found that stem cells isolated from females showed higher levels of cardioprotective factors than those isolated from males and conferred greater cardioprotection from I/R injury when infused into isolated hearts. Estrogen-conditioned stem cells showed increased secretion of VEGF and SDF-1α, reduced expression of caspase 3 and attenuated activation of STAT3 [82]. Infusion of estrogen-treated cardiac stem cells into isolated mouse hearts before I/R injury reduced myocardial damage and improved cardiac function [82–85]. These effects were achieved in the absence of stem cell differentiation [83].

In a carotid artery injury model in ovariectomized mice, estrogen promoted re-endothelialization of injured vessels and prevented neointimal formation [73]. Similarly, in a hind limb ischemia model, estrogen increased capillary density and limited ischemic tissue injury [70]. The vasculoprotective effects of estrogen in this model was associated with increased EPC proliferation, mobilization, and migratory capacity and reduced apoptosis.

A role for androgens in influencing cardiomyocyte regeneration after ischemic injury has not received much attention [86, 87]. Embryonic stem cells (ESCs) express AR. Testosterone stimulates differentiation of these cells toward cardiac lineage. Exposure of murine embryonic bodies to androgen increases the expression of cardiac markers and increases the number of beating cardiomyocytes. It is unclear whether testosterone's ability to enhance cardiomyocyte differentiation is mediated by classical AR signaling pathways or via activation of calcium channels.

The influence of testosterone on the biology of EPCs has not been well studied and the data are conflicting. Despite the fact that EPCs express AR, a number of studies found no influence of testosterone on the number of circulating EPCs [88, 89]. However, other studies found that testosterone increases EPC proliferation, migration, and colony formation capacity [90, 91]. In a hind limb ischemia model in which bone marrow from male mice was transplanted into irradiated female mice, Lam et al. [92] showed that testosterone increases bone marrow EPC production, mobilization, and homing to ischemic tissue and reduces cellular apoptosis. Testosterone-treated animals showed increased production of proangiogenic cytokines and increased incorporation of EPCs into newly formed vessels. Interestingly, in another model, androgens improved angiogenesis in male but not in female animals, but androgen sensitivity was conferred in females overexpressing AR [93].

Muscle

Skeletal muscle is an extremely stable tissue under normal circumstances, showing very little cell turnover [94]. Resident skeletal muscle satellite cells are skeletal muscle precursor cells that play a major role in the regulation of muscle growth and regeneration [95]. Activation and entry of these cells into the cell cycle from their basal quiescent state occur after muscle injury [96]. After activation, satellite cells undergo asymmetric cellular proliferation and differentiate into myoblasts, which undergo further transformation and fuse to form new myofibers [96]. As shown in Fig. 22.3, satellite cell biology and the involvement of these cells in muscle repair and regeneration are strongly influenced by sex hormones. ERα has been identified as being necessary for satellite cell self-renewal and muscle regeneration after

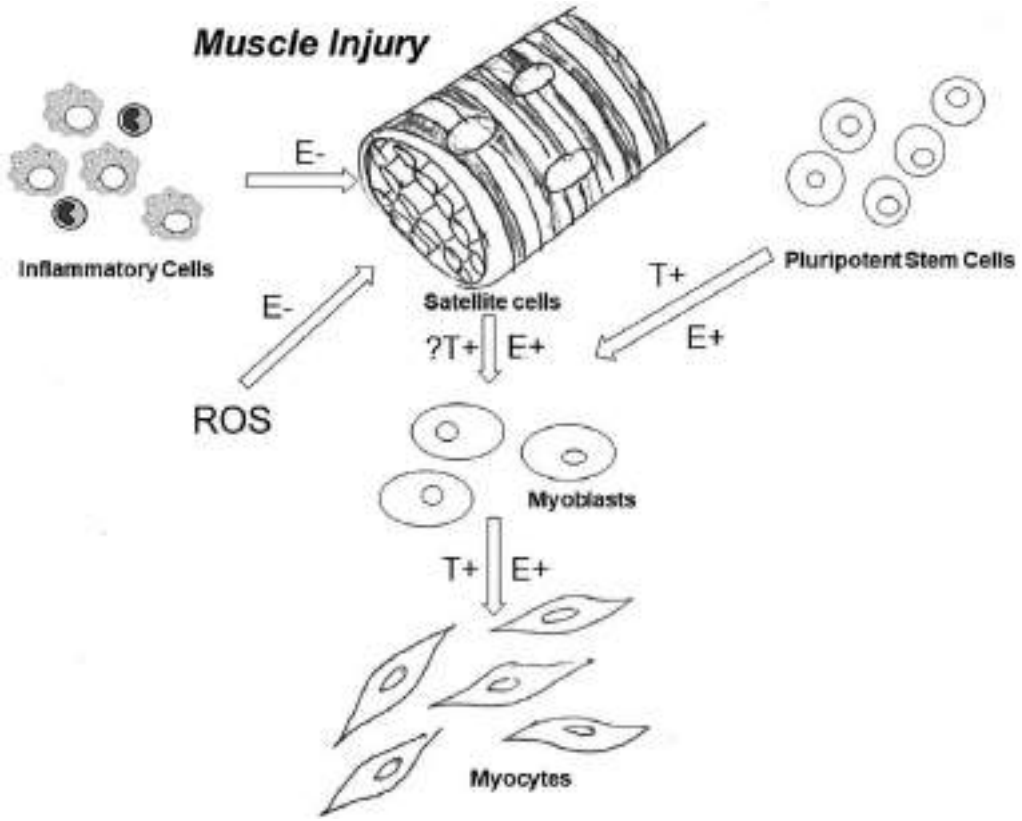

FIG. 22.3 Mechanisms by which sex hormones influence repair and regeneration after skeletal muscle injury. Abbreviations: *E +*, estrogen stimulates the pathway; *E −*, estrogen inhibits the pathway; *ROS*, reactive oxygen species; *T +*, testosterone stimulates the pathway.

injury [96]. Estrogen deficiency, associated with menopause or induced by ovariectomy, is associated with a reduced number of resident satellite cells, compromise of satellite cell self-renewal and impaired differentiation into myoblasts [96]. It has been further suggested that estrogen promotes satellite cell activation by upregulating the PI3K/Akt signal transduction pathway [95]. Ovariectomy increases satellite cell apoptosis via ERα-dependent mechanisms which upregulate genes associated with caspase-dependent apoptosis including p53 and caspase-3 [65].

Female mice show a smaller increase in creatine kinase levels after muscle-damaging exercise compared with male, ovariectomized female, or ERβ KO mice [97, 98]. Estrogen deficiency impairs recovery of muscle strength after injury or immobilization, an effect that is reversed by estrogen supplementation [94, 96–99]. Estrogen promotes muscle repair after injury by several mechanisms, including activating satellite cells, stabilizing the integrity of muscle membranes, reducing oxidative stress, and downregulating the inflammatory response to injury [99]. Estrogen attenuates the inflammatory response to muscle injury after I/R and after exercise-induced muscle damage, in part, by decreasing the recruitment of neutrophils, macrophages, and monocytes to sites of injury [95]. The ability of estrogen to reduce leukocyte infiltration is mediated by reduced expression of NFκB and increased expression of the antiinflammatory cytokine, IL-10 [97, 98]. Velders et al. [97, 98] have suggested that ERβ is the isoform primarily responsible for upregulation of cytoprotective and antiinflammatory cytokines, satellite cell activation and accelerated muscle regeneration. However, other investigators attribute the beneficial effects of estrogen to ERα, while still other investigators have implicated both receptors [95, 96].

Although male and female pluripotent stem cells possess a similar capacity to differentiate, smooth muscle progenitor cells derived from these cells exhibit sexual dimorphism [100]. In studies performed by Li et al. [100], treatment with estrogen stimulated cellular proliferation and led to a robust increase in myogenic gene markers associated with reduced MMP expression and increased TIMP expression in smooth muscle progenitor stem cells derived from female but not male donors. In a rat myoblast cell line, Galluzzo et al. [94] found that the ability of estrogen to promote myogenic differentiation was mediated by multiple pathways including ERα-dependent Akt activation and ERβ-dependent p38 activation. Estrogen has also been shown to promote the differentiation of adipose cell-derived stem cells into cells of myocyte lineage [101].

Testosterone promotes regeneration after muscle injury by activating resident satellite cells and increasing muscle protein synthesis via AR-dependent mechanisms [97, 98]. Testosterone increases myofiber cross-sectional area in regenerating muscle [99]. Although data concerning the effects of testosterone on satellite cell biology are inconsistent, most studies show enhanced satellite cell proliferation and an increase in the number of these cells in injured muscle after exposure to testosterone. Testosterone rapidly activates these cells, inducing them to transition from a quiescent state to enter the cell cycle, proliferate, and differentiate into myofibers [102]. MacKrell et al. [102] employed gene expression profiling to show that the ability of androgens to promote muscle regeneration was associated with increased markers of satellite cell activation (increased IGF-1 and a transient increase in hepatocyte growth factor), migration (a transient increase in CXCR4), and growth (increased follistatin). In contrast, androgen deprivation was associated with delayed muscle regeneration and prolonged upregulation of TNFα. Testosterone also promotes the commitment of pluripotent stem cells to myogenic lineage and inhibits differentiation toward cells of adipogenic lineage via AR-dependent mechanisms [103]. Fu et al. [104] have suggested that testosterone's ability to increase proliferation and differentiation of myoblasts is cell line specific, mediated by classical AR pathway in some lines and by G-protein-coupled receptor pathways in others; in the latter case, stimulation of proliferation is mediated by PKC-p44/42 MAPK signaling while stimulation of differentiation is mediated by PKA signaling.

Testosterone-induced muscle hypertrophy is dependent on increased protein synthesis [102, 105]. In this regard, testosterone increases the expression of genes encoding growth promoting factors. The ability of testosterone to increase muscle size in models of muscle atrophy is mediated by increased expression of IGF-1 [102]. Activation of PI3K/Akt and follistatin signal transduction pathways also contribute to an increase in protein synthesis and a decrease in protein degradation.

Liver

Estrogen promotes regeneration after liver injury. Hepatocytes primarily express ERα, whereas cholangiocytes and hepatic stellate cells primarily express ERβ [106]. Most studies have shown that physiologic concentrations of estrogen stimulate the proliferation of hepatocytes in vitro, an effect that is reversed by estrogen receptor antagonism [107–109]. Estrogen promotes liver regeneration, stimulates cellular proliferation, and increases liver weight after subtotal hepatectomy. Subtotal hepatectomy is associated with a rapid increase in serum estrogen levels, a decline in serum testosterone levels, increased hepatic ER density, and reduced AR density [108]. A temporal association was observed between estrogen receptor translocation from a cytosolic to a nuclear location and increased hepatic cellular proliferation, consistent with hypothesis that the proliferative response is mediated by ER-dependent mechanisms [108, 110]. Administration of an estrogen receptor antagonist leads to a reversal of the proliferative response to partial hepatectomy [107]. Estrogen also upregulates IL-6 after subtotal hepatectomy, and it has been suggested that this cytokine may mediate the beneficial effects of estrogen on liver regeneration.

Kao et al. [111] showed that liver regenerative capacity was reduced in both ERα KO and ERβ KO mice. ERα KO mice showed decreased proliferation in regenerating liver, whereas ERβ KO mice showed decreased cellular differentiation. The investigators found that the level of ER expression, not circulating estrogen levels, correlated with regenerative capacity. These investigators went on to analyze and compare transcriptomes and interactomes from regenerating liver from ERα KO, ERβ KO, and wild-type mice after subtotal hepatectomy. The ERα-associated pathways were involved in promoting cellular proliferation, whereas the ERβ-associated pathways regulated metabolic function and were involved in promoting cellular differentiation into hepatocytes and cholangiocytes. Tsugawa et al. [112] also demonstrated impaired liver regenerative capacity in ERα KO mice, which was attributed to attenuate upregulation of TNFα and IL-6 and enhanced activation of NFκB and STAT3.

Estrogen reduces liver damage and increases survival after hepatic I/R injury, an effect that has been attributed to upregulation of eNOS activity via nongenomic mechanisms [113]. In a model where a fragment of liver is transplanted under the kidney capsule of mice, estrogen increased the proliferation of regenerating hepatocytes, sinusoidal endothelial cells, and Kupfer cells [114]. In addition, estrogen accelerated vascularization and stimulated reticuloendothelial function. In another model, involving hepatic artery partial branch ligation, pharmacologic blockade of ERα impaired hepatic regeneration [106]. The investigators suggested that the effects of estrogen on liver regeneration were mediated by upregulation of eNOS resulting in increased hepatic blood flow. Other investigators concurred that the beneficial effects of estrogen in this model were due to increased hepatic blood flow, but identified increased serotonin as the mediator [115].

Nervous system

Peripheral, spinal, and cranial nerves

Estrogen and androgen receptors are widely distributed throughout the peripheral nervous system, brain, and spinal cord [116]. Sex hormones have been shown to have neurotropic effects, to enhance nerve cell survival, growth, and metabolism, and to promote neuronal differentiation, synaptogenesis, and elaboration of cell processes [116, 117]. Sex hormones also accelerate peripheral, spinal, and cranial motor neuron repair and regeneration after nerve injury [116–129].

Injured peripheral, cranial, and spinal nerves are capable of regenerating, but the capacity for repair is limited and functional recovery is poor [130]. Pharmacologic doses of testosterone, administered at the time of nerve injury, accelerate the rate of axon regrowth and reinnervation of target muscle tissue and enhance functional recovery after peripheral or spinal nerve crush injury in male hamsters via AR-dependent mechanisms [116–129]. Testosterone preserves synaptic contacts normally lost following injury and enhances neurite outgrowth and axon regeneration associated with upregulation of cytoskeleton-associated proteins including β_{II} tubulin [116]. Testosterone also stimulates the production of growth-promoting and neurotrophic factors that enhance axon regrowth and synaptogenesis [131]. These effects are mediated by upregulation of genes that reduce oxidative stress, inhibit cellular apoptosis, and regulate microtubule assembly and actin polymerization [131, 132]. In addition, testosterone regulates the microglia response to peripheral nerve injury by downregulating reactive gliosis and astrocyte proliferation and stimulating the production and secretion of trophic cytokines and growth factors [116, 133].

Jones et al. [116] have postulated that the ability of testosterone to accelerate neural repair and regeneration results from modification of the stress response to injury. Nerve injury is associated with increased transcription of ribosomal RNA, but the time interval between increased transcription and the resultant increase in ribosomal units is prolonged [116]. Testosterone reverses this delay, an effect that may be mediated by interactions between AR and heat shock protein 70 [120].

Although the benefits of exogenous testosterone administration on neural regeneration after crush injury are reduced in female hamsters compared with males, untreated female hamsters showed faster neural regeneration after crush injury than do males [118]. Female animals also show a greater capacity for intact neurons surrounding an area of injury to develop collateral sprouts to reinnervate target muscle [75, 134, 135]. In this context, administration of estrogen accelerates axonal regeneration and recovery via ER-dependent mechanisms in various models including nerve crush injury and peripheral nerve transection [130, 136]. Accelerated repair was associated with increased expression of both ER isoforms [130]. Estrogen increases the number of axons undergoing regeneration and increases axon length [130]. Estrogen also reduces neuronal apoptosis associated with Akt kinase activation [117]. In addition, estrogen increases angiogenesis and capillary density after nerve crush injury, in part, due to upregulation of sonic hedgehog-mediated angiogenesis [137]. Importantly, the beneficial effects of estrogen on neural tissue are replicated by selective estrogen modulators, which lack the adverse effects of estrogen on reproductive tissues [138]. In contrast, other investigators have minimized the role of the hormonal milieu and have attributed sexual dimorphism in neural regeneration to sex chromosome-related differences in axon growth capacity [134, 135].

Estrogen promotes differentiation of neural stem cells into mature endothelial cells, stimulates endothelial cell proliferation and migratory capacity, and reduces cellular apoptosis, which in turn contributes to enhanced angiogenesis at sites of tissue injury. In a murine model in which neural stem cells isolated from newborn brain underwent intraneural transplantation into injured sciatic nerves, estrogen enhanced neurogenesis and functional recovery, associated with increased VEGF levels, enhanced angiogenesis, and increased tissue perfusion [117].

Central nervous system

Women are protected from ischemic stroke until the onset of menopause [117]. Moreover, numerous observational studies suggest that estrogen replacement therapy administered to postmenopausal women affords cerebrovascular protection [117]. Similarly, in experimental models of ischemic stroke, estrogens exert unequivocal neuroprotective effects [117]. However, results from The Womens' Health Initiative suggest the opposite [139]. Estrogen therapy increased the risk of first stroke and failed to improve stroke outcome [139]. The explanation for these contradictory observations is unclear.

In an experimental model of middle cerebral artery occlusion, physiologic doses of estrogen are neuroprotective [117]. However, infarct volume was reduced in this model only when estrogen was administered prior to injury. In contrast, other investigators have demonstrated neural protection from ischemic brain injury up to 6 h after the ischemic insult when *supraphysiologic* doses of estrogen are administered [117]. Estrogen protects neurons in the ischemic penumbra from apoptotic cell death. Neuroprotection from ischemia is associated with attenuated downregulation of Bcl-2 and attenuated upregulation of caspase 3 and early response genes [117]. Ischemic injury upregulates ERα

but not ERβ in the cortex of ovariectomized animals, and the neuroprotective effects of estrogen are lost in ERα KO mice suggesting that the ERα is responsible for neuroprotection from ischemic stroke [117].

Multiple mechanisms contribute to the protective effects of estrogen in experimental ischemic cerebral injury. Estrogen enhances neurogenesis after ischemic injury [117]. Estrogen possesses antiinflammatory properties and attenuates peripheral and intracerebral upregulation of proinflammatory and vasoactive cytokines [117]. Both isoforms of ER contribute to the antiinflammatory effects of estrogen, but the contribution of ERα is more significant. Upregulation of iNOS is critical to the inflammatory response associated with ischemic brain injury and mediates estrogeńs neuroprotective effects insofar as estrogen is no longer neuroprotective in iNOS KO mice [140]. In addition, estrogen inhibits microglial activation and IL-6 expression in these models.

Sex hormones influence neural stem cell proliferation and differentiation in vitro; however, these effects are modified by the presence or absence of other growth factors in the media and the age of the stem cell [141–144]. Estrogen alone stimulates the proliferation of embryonic stem cells in an ER-dependent manner but does not affect the proliferation of adult stem cells which express a lower ratio of ERα to ERβ [141–144]. Estrogen preferential stimulates differentiation of neural embryonic stem cells into neurons relative to glial cells, increasing the ratio of neurons to glial cells [141–143]. The proliferative effect of estrogen on these cells has been attributed to classical genomic ER pathways, whereas effects on differentiation have been attributed to MAPK activation via nongenomic pathways involving membrane-bound G-protein-coupled estrogen receptors [142, 143, 145].

The forebrain subventricular zone is the main repository of neural stem cells in the adult brain [146]. After cerebral injury, these cells are mobilized to the area of injury to promote regeneration. At the site of injury, subventricular zone-derived neuroblasts differentiate into mature neurons. However, the number of recruited cells is inadequate to effectuate recovery [117, 146]. Physiologic doses of estrogen stimulate the proliferation of neural stem cells and enhance neurogenesis following ischemic stroke, an effect that is abolished in both ERα and ERβ KO mice [146]. It has been suggested that the ability of estrogen to promote proliferation and differentiation of these cells is primarily mediated by suppression of inflammatory cytokines [117, 146].

Estrogen also stimulates neural differentiation and maturation of human umbilical cord blood mesenchymal cells [147, 148]. Estrogen enhances neurogenesis and stimulates neurite branching in these cells [147, 148]. It has been suggested that these effects are in part mediated by increased expression of neurotrophic growth factors [147–149].

Investigators have been able to reprogram fully committed differentiated cells from one lineage to another after exposure to gonadal sex hormones [150, 151]. The combination of progesterone, testosterone, and estrogen, but not any of these agents alone, induces transdifferentiation of ovarian surface epithelia and follicular granulosa cells into neural stem cells and differentiating neurons [151]. These investigators also showed that the same sex hormone combination can induce transdifferentiation of vascular smooth muscle cells into neural cells [150].

Testosterone also plays a role in neuron regeneration after cerebral injury. Bramble et al. [152, 153] showed that exposure of murine embryonic neural stem cells to testosterone altered the expression of numerous genes including those involved in DNA methylation and histone acetylation. They also found differences in gene expression in testosterone-treated ESCs of male versus female origin, suggesting that both chromosomal differences between the sexes and differences in the hormonal milieu influence sexual dimorphism in gene responses. ARs are also expressed on astrocytes and oligodendrocytes, which participate in remyelination after demyelinating injury [154]. In this regard, testosterone stimulates myelin regeneration in murine models of demyelinating disease. Testosterone enhances recruitment of astrocytes, which are necessary for oligodendrocyte-mediated remyelination, and promotes the differentiation of oligodendrocyte progenitor cells into mature oligodendrocytes capable of producing myelin-associated proteins.

Conclusion

Sexual dimorphism in regeneration and recovery from tissue injury has been convincingly demonstrated. Sufficient evidence as to the mechanisms and direction of these differences in the kidney is still lacking. The effects of sex hormones in accelerating repair and regeneration of cardiac, musculoskeletal, hepatic, and neural tissue might be exploited to enhance functional recovery after renal injury. Future directions should utilize a translational approach to exploit any enhanced regenerative potential observed after sex hormone administration.

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PART V

Kidney engineering

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CHAPTER

23

Glomerular stem cells☆

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Introduction

Adult stem cells (also known as somatic stem cells or tissue stem cells) are rare populations of unspecialized cells that are found in the body throughout the majority of postnatal life [1]. Two essential functional features define adult tissue stem cells [2]. First, a population of tissue stem cells should be maintained over long periods of time, often the entire lifetime of the organism and undergo self-renewal, which is the ability to go through numerous cycles of cell division while maintaining indefinitely an undifferentiated state to support the maintenance of the stem cell reservoir [3]. Second, these long-lived stem cell populations should be able to generate the differentiated cell types of the tissue [4]. Most adult stem cells can generate different cell types, but also unipotent stem cells have been described. Progenitor cells are distinguished from stem cells usually for a more limited potency and self-renewal capacity [5]. Thanks to these functional features, their progeny replaces cells that are lost during homeostasis and tissues turnover or following injury [5]. Resident stem cells have been identified in different tissues in mammals and well-studied examples include blood [6], skin [7], intestine [2], and muscle [8]. In the last decade, many groups performed intensive studies to isolate kidney stem cells and progenitors from adult mammalian kidney and interest and research into how resident adult stem cell populations in the kidney behave during homeostasis and how they respond to injury have been dramatically increased [9–20].

The renal glomerulus is a very specialized structure that operates as a perfectly selective plasma filtration unit [21]. Three cell types compose the glomerulus, the endothelial cells, the mesangial cells, and the podocytes, and this composition is conserved from the primitive kidneys of fishes and amphibious vertebrates to the more advanced kidneys of birds, reptiles, and mammals [22]. The normal development and integrity of the glomerulus are essential to maintain the efficient removal of metabolic/exogenous waste products without inadvertent loss of useful substances, such as plasma proteins and blood cells into urine [21]. Many researchers, for decades, considered the glomerulus incapable of regeneration. However, in recent years, the concept that regression of glomerular injury and even glomerulosclerosis can be achieved has been intensely discussed, and a growing body of experimental and clinical evidence documents this phenomenon under certain circumstances [23–28]. The critical problem with glomerular regeneration is on the podocyte. Indeed, experimental data and the discovery that mutation of podocyte-specific proteins can cause genetic glomerular diseases in humans establish the podocyte as central to normal glomerular anatomy and function, and support a major role for podocyte damage in loss of glomerular function in a variety of glomerular diseases [29]. In contrast to endothelial and mesangial cells, which following injury are simply replaced through proliferation of the remaining cells, podocytes are terminally differentiated and highly specialized cells indefinitely arrested in the G0 phase of the cell cycle, unable to proliferate [30]. They are still able to undergo limited DNA synthesis, but to try to enter cell division they have to reorganize the actin cytoskeletal fibers, essential for maintaining the complex, arborized network of foot processes, and consequently their morphological stability and cell-matrix adherence [30]. Therefore, the consequences of the changes in the complex cytoskeletal architecture of the foot processes are podocyte effacement, cell detachment, or death by mitotic

☆ Cover illustration is processed with Fig. 23.1 (Chapter 23 by L. Lasagni, B. Mazzinghi, and P. Romagnani).

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catastrophe, with disruption of the glomerular filtration barrier (GFB) and ensuing proteinuria [30, 31]. Podocyte hypertrophy that is an increase in cell size in an attempt to cover the underlying glomerular basement membrane in exposed areas where neighboring cells have detached or died can compensate for an initial loss of podocytes, lower to 20% [32]. Further progressive loss of 20%–40% of podocytes is associated with a point of no return that results in scarring and progressive sclerosis [32]. Several clinical observations support the potential of glomerular regeneration. Indeed, remission and regression of functional parameters of chronic kidney disease can occur in patients with diabetic and nondiabetic nephropathies [28, 33, 34]. Studies using nongenetic cell fate mapping in experimental models of diabetic and nondiabetic kidney disease with reduction in podocyte number showed that the podocyte number could be restored in absence of podocyte proliferation [35, 36]. More clearly, lineage tracing studies in conditional transgenic mice conclusively demonstrated the possibility of structural lesion regression with generation of novel podocytes, when the podocyte depletion after injury was below 20% [13, 19, 37, 38]. Finally, using intravital imaging, Hackl and Peti-Peterdi provided direct evidence for podocyte regeneration in experimental models of glomerular disease [39]. Serial multiphoton microscopy of the same injured glomerulus revealed the appearance of a new podocyte (labeled with a fluorescent reporter) within 24 h of the previous image. Taken together, these results strongly support the notion that following a decrease in absolute podocyte number in experimental mouse models, replacement of podocytes can occur at least partially. The absence of podocyte proliferation is consistent with a nonpodocyte source for their replacement, which is a podocyte stem/progenitor pool.

Glomerular progenitor cells

The first cells proposed as being able to replace podocytes were bone-marrow-derived cells due to their capacity to differentiate into a wide range of cell types. Initial studies suggested that extra-renal cells contribute to the repair of renal tissues following kidney injury [40, 41]. More recent studies have shown the incapacity of bone-marrow-derived mesenchymal cells to differentiate into epithelial cells and argue that their beneficial effects in kidney diseases are rather limited to the secretion of regenerative and prosurvival paracrine factors [42–44]. We first reported the existence of a kidney intrinsic source of progenitors denominated renal progenitor cells (RPCs) and demonstrated their capacity to replace injured podocytes in an adult human kidney [11, 12]. We identified these cells through the assessment of the presence of both CD24, a surface molecule that has been used to identify different types of human stem cells [45, 46], and CD133, a marker of several types of adult tissue stem cells (Fig. 23.1A) [47]. A subset of parietal epithelial cells (PECs) localized at the urinary pole of Bowman capsule in adult kidneys and characterized by self-renewal, a high clonogenic efficiency, and multidifferentiation potential expressed both markers. We further described RPCs to form a heterogeneous and hierarchical population of undifferentiated cells, including multipotent cells and an intermediate population of transitional cells—or podocyte progenitors—expressing both podocyte and progenitor markers and capable of differentiating into podocytes [12]. When injected into severe combined immunodeficiency mouse models of focal segmental glomerulosclerosis (FSGS), human CD133 + CD24 + RPCs could engraft, replace lost podocytes, and improve proteinuria, suggesting that RPCs can differentiate into podocytes and promote podocyte regeneration. These findings obtained in human kidneys were confirmed in a parallel study performed in murine kidney by Appel et al. [9], who also detected transitional cells with morphological and immunohistochemical features of both PECs and podocytes in proximity of the glomerular vascular stalk. More importantly, using genetic tagging of PECs in rodents, the same authors demonstrated that podocytes are recruited from PECs, which proliferate and differentiate from the urinary to the vascular stalk, then generating novel podocytes [9]. This occurs as the kidney grows, during childhood and adolescence [9], and may take place following an injury that allows a slow, regulated generation of novel podocytes, such as uninephrectomy. Wanner et al. [37] validated the role of PEC-derived podocytes to the population of glomerular tuft during adolescence and suggested that this podocyte reservoir generates around 10% of the podocytes present in the adult glomerulus during postnatal kidney growth. This fits with recent studies in humans by Puelles and Bertram, showing that the number of podocytes increases by up to 20% early in life [48]. We established models to trace the mouse counterpart of human RPCs within Bowman capsule making use of the regulatory region of the transcription factor Pax2, a critical regulator of the embryonic progenitor population during kidney development [49], and of fluorescent reporters for the generation of transgenic mouse models useful for lineage tracing and clonal analysis of progenitors (Fig. 23.1A) [13]. With these models, we demonstrated that ~ 8%–10% of total podocytes observed in the adult mouse kidney are derived from PAX2 + PECs after birth. Moreover, PAX2 + PECs behave as podocyte progenitors, even once glomerular development has ended and contribute to the increase of kidney dimension that is observed during childhood and adolescence. In adult animals, PAX2 + cells remained

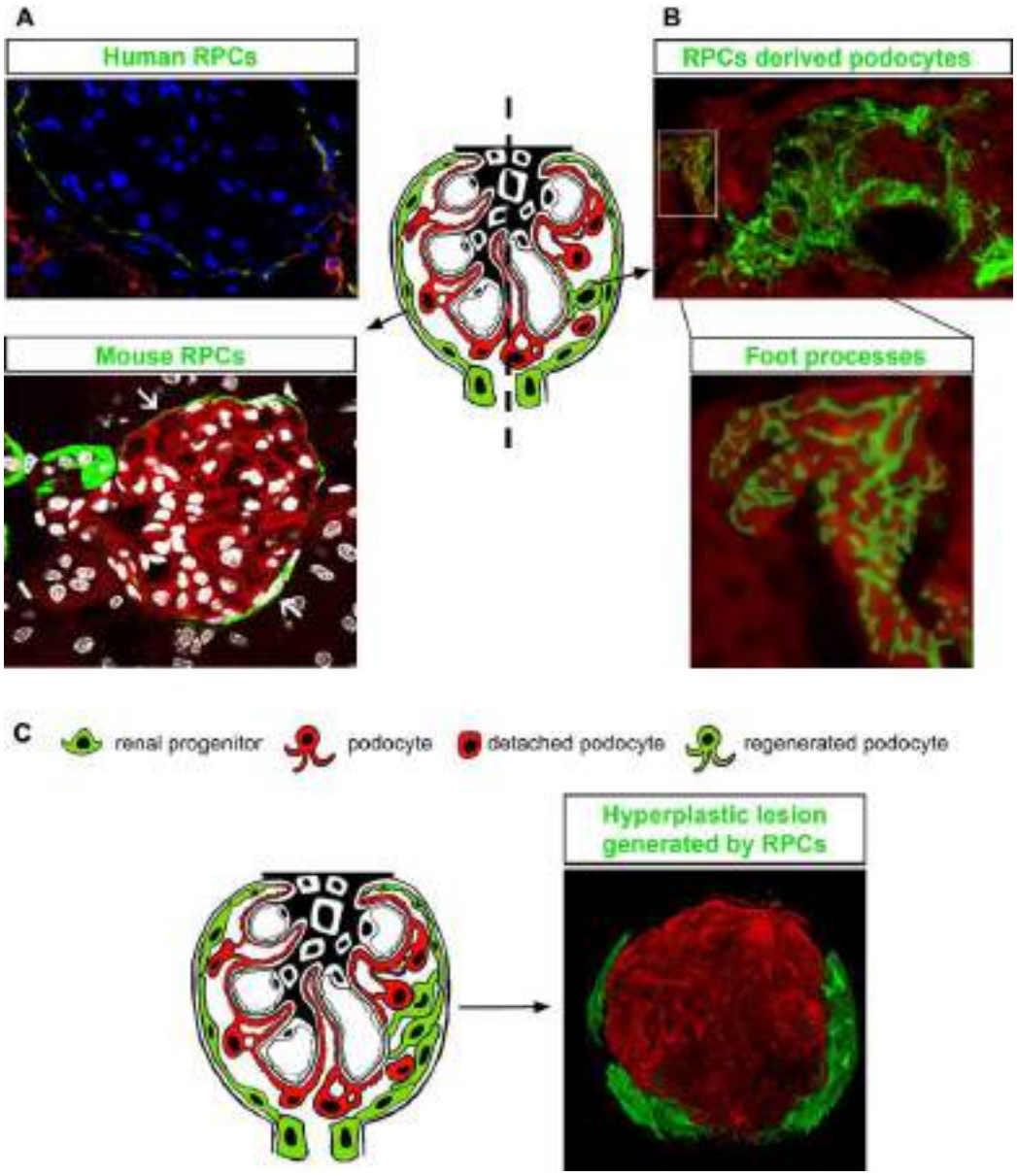

FIG. 23.1 (A) In healthy glomeruli, CD133 + CD24 + progenitor cells, in human (*top*), and Pax2 + cells, in mouse (*bottom*), are localized within PECs of Bowman capsule. (B) Following glomerular injury, RPCs can generate differentiated podocytes. Representative deconvolved STED image of a Pax2-derived podocyte (green cells, *top*), and drawing of the boxed area to better visualize the foot processes wrapping the capillaries (*bottom*). (C) After massive podocyte injury, dysregulated renal progenitors generate hyperplastic lesions. The panel shows a 3D reconstruction of a glomerulus with lesion generated by Pax2 + cells in mouse affected by crescentic glomerulonephritis. GFP stains Pax2 + cells (*green*) and synaptopodin stains podocytes (*red*). *Adapted from Romoli, S. et al. CXCL12 blockade preferentially regenerates lost podocytes in cortical nephrons by targeting an intrinsic podocyte-progenitor feedback mechanism. Kidney Int 94, 1111–1126, https://doi.org/10.1016/j.kint.2018.08.013 (2018) licensed under a Creative Common Attribution 4.0 International License, and Lasagni, L. et al. Podocyte regeneration driven by renal progenitors determines glomerular disease remission and can be pharmacologically enhanced. Stem Cell Rep 5, 248–263, https://doi.org/10.1016/j.stemcr.2015.07.003 (2015) licensed under the terms of the Creative Commons CC-BY license.*

mostly localized within the Bowman capsule of subcapsular glomeruli, but in only ~ 30% of juxtamedullary glomeruli [13]. Using a dual-reporter transgenic mouse, Kaverina et al. provided strong evidence that PECs serve as a source of new podocytes in adult mice demonstrating that a subset of PECs differentiate to a podocyte fate after podocyte depletion in experimental FSGS[19] and in aged kidney [50].

In embryonic human kidneys, coexpression of CD133 and CD24 characterized a subset of cells in the cap mesenchyme, renal vesicles, and S-shaped bodies that displayed self-renewal and multidifferentiation potential [51]. During nephron development, CD133 + CD24 + cells progressively disappeared until they remained selectively localized to

cells of the urinary pole of Bowman capsule [51]. When injected into mice with acute kidney injury, CD133 + CD24 + renal embryonic stem cells regenerated cells of different portions of the nephron, reduced tissue necrosis and fibrosis, and significantly improved renal function [51]. In agreement with their nature, CD133 + CD24 + renal embryonic stem cells also expressed high levels of the stem-cell-related transcription factor BmI-1, a member of the polycomb group of transcription repressors that is necessary for efficient self-renewal in hematopoietic and neural stem cells [52, 53]. This supports the concept that CD133 + CD24 + renal stem cells directly derive from renal embryonic stem cells and the urinary pole of Bowman capsule represents a stem cell niche, which is a specific site in adult tissues where stem cells reside.

Molecular pathways regulating glomerular progenitor cells in homeostasis and in response to injury

The discovery that renal progenitors of Bowman capsule represent a potential source for podocyte generation provides the basis for a novel concept that injured podocytes can be replaced. The possibility that progenitors localized within PECs can migrate from Bowman capsule to the capillary tuft in areas different from the vascular pole was previously suggested by some studies, which proposed that areas of adhesion, as well as bridges between Bowman capsule and the glomerular tuft may represent such sites of migration [54, 55]. A recent study using genetic tagging of PECs demonstrated that in experimental models of glomerular disorders PECs generate cell bridges between Bowman capsule and the glomerular tuft [56]. This may provide a slide for the migration and differentiation of an adjacent progenitor and a quick replacement of lost podocytes, or alternatively bridging PECs may acquire podocyte markers following injury [57], and directly replace the lost podocytes. In summary, a large body of evidence indicates that Bowman capsule of adult kidneys contains a population of renal stem cells that can replace lost podocytes through multiple mechanisms and allow glomerular regeneration (Fig. 23.1B).

However, as in other adult organs, a response to injury can shift the balance from regeneration to repair depending on a complex interaction between the stem cell compartment and the surrounding environment [58–60]. In normal conditions, stem cells respond to injury in a strictly regulated manner. Because of resident stem cell depletion and/or uncontrolled growth, healing problems can manifest in every injured tissue and induce previously functional tissue to become a patch of cells and disorganized extracellular matrix that is referred to as a scar [58–60]. Therefore, at least in proteinuric glomerular disorders, we could envision glomerulosclerosis as a scar induced by insufficient or exaggerated responses of renal stem cells to podocyte injury.

Extracapillary proliferative lesions or crescents are the hallmark of both inflammatory and noninflammatory glomerular diseases. Evidence in experimental models [56, 61] and in human biopsies indicates that crescents are composed of PECs [62] that abnormally shift their reaction from reparative to injurious (Fig. 23.1C). It is not completely understood which factors are responsible for this tilting the unbalanced activation. Crescentic glomerulonephritis (CGN) is the best-characterized disease in which PECs are the major culprits. Cellular crescent, defined as the multilayered accumulations of PECs and other cell types within Bowman space, is the typical morphological change observed in CGN. Consequently, it occludes the urinary outlet and the flow of the primary urine, and later the implicated nephron is impaired. The rupture of glomerular capillaries in crescentic disease leads to the exposure of PECs to high concentration of plasma that dramatically increases the proliferation of murine and human PECs in culture [63]. Several plasma components could account for this PEC hyperplasia but, currently, there are consistent data only for fibrinogen activation, a member of the activated coagulation cascade during vascular injuries. A lack of fibrinogen or fibrinolysis prevents PEC hyperplasia formation in several rodent models of glomerular crescent formation [63, 64].

Taken together, these results support the concept that the outcome of glomerular disorders may depend on a strictly regulated balance between the degree of podocyte injury and the amount of regeneration provided by renal stem cells. The identification of the pathways that regulate a correct proliferative and differentiative response of RPCs during homeostasis and following injury is a critical obstacle that researchers should overcome to design modulators of RPC regenerative potential.

- *β-catenin/Wnt signaling*. Recent studies on kidney development demonstrated that β-catenin/Wnt signaling is an important mechanism that controls later stage of nephrogenesis and lineage specification of PECs. The conditional β-catenin knockout mice in late S-shaped bodies showed a normal tubular system along with a lacking outermost cortex, and abnormal glomeruli with underdeveloped capillary tufts and transformation of PECs into podocytes. In particular, the use of conditional β-catenin knockout mice allowed establishing that β-catenin/Wnt signaling is involved in PEC fate decision, and in its absence, parietal epithelial precursor cells direct switch to "parietal podocytes," which can no longer undergo cellular division [65, 66].
- *Noncoding RNAs*. miR-193 is a master switch in the differentiation of PECs toward podocytes and during development, PECs express high levels of miR-193 compared with podocyte. In vitro studies demonstrated that,

following in vitro miR-193 knockdown, PECs acquire a podocyte-like phenotype, developing prominent cortical F-actin ring with centripetal F-actin fibers, and increased expression of podocyte specific markers as WT1, Podocalyxin, Synaptopodin, α-Actinin-4, and NPHS1. Of note, the knockdown of miR-193 in PECs resulted also in a decreased expression of the progenitor markers, CD24 and CD133 [67]. In addition, in vitro studies demonstrated that Apolipoprotein L1 (APOL1) controls PEC phenotype through miR-193 modulation. APOL1 and miR-193 displayed a reciprocally linked feedback loop. In basal condition, human PECs did not express APOL1 because of their high constitutive expression of miR-193a. Nonetheless, downregulation of miR-193a in PECs not only induced APOL1 expression but also stimulated PECs to acquire a podocyte molecular phenotype. Because miR193a inversely regulated the expression of APOL1, loss of APOL1 seemed to be the trade-off by PECs to preserve the integrity of Bowman capsule in high miR-193 milieu. The high expression of miR193 not only downregulated APOL1 and WT1 expression but also enhanced Pax2 expression, a marker of PECs with progenitor features [68, 69].

- *Notch pathway*. The canonical Notch signaling pathway is a conserved mechanism that regulates cell decision to proliferate and/or to differentiate. In CD133 + CD24 + PECs, Notch activation lead to entry into S-phase of cell cycle and successive cell division, while its downregulation induced upregulation of p21, p27 p57, and downregulation of cyclin D1, which are essential for CD133 + CD24 + PEC differentiation in podocyte. However, defective downregulation of the Notch pathway during CD133 + CD24 + PEC differentiation determined the formation of podocyte with abnormal DNA content and their subsequent death by mitotic catastrophe. Accordingly, inhibition of Notch during the regenerative phase in a murine Adriamycin nephropathy model resulted in worsening of albuminuria and glomerulosclerosis. Furthermore, prolonged Notch blockade in a transgenic mouse model of FSGS decreased PEC proliferation and formation of hyperplastic lesions but worsened podocyte loss and proteinuria [70]. Therefore, a tight regulation of the Notch signaling mediates PEC behavior to compensate for podocyte loss in filtration barrier. Taken together, these evidences show that Notch is a key regulator not only of kidney development but also of glomerular repair [71].
- *CXCL12/CXCR4*. Recent studies suggested that podocyte-derived CXCL12 suppresses Notch signaling in glomerular progenitors of Bowman capsule and that this mechanism maintains their quiescence during homeostasis. Furthermore, lineage tracing of glomerular progenitors in mice demonstrated that CXCL12 inhibition boosts podocyte regeneration in Adriamycin nephropathy reducing proteinuria and glomerulosclerosis. Quantitative 3D analysis and STED super resolution microscopy of injured glomeruli showed that a significant increase in the number of new fully differentiated podocytes perfectly integrated in the GFB and derived from renal progenitors of Bowman capsule (Fig. 23.1B) supports the disease improvement [72].
- *Retinoids*. Among other factors involved in the regulation of glomerular progenitor differentiation toward podocyte linage are retinoids. Several lines of evidence indicated that retinoid signaling is crucial for nephron development modulating, directly or indirectly, renal gene expression by nephron progenitors [73, 74]. In addition, recent studies suggested that retinoic acid (RA) participates actively in triggering gene expression in podocyte lineage. Interestingly, functional RA-responsive elements (RARE), which mediate responsiveness to RA in vivo, are present in the promoter region of WT1 gene [75]. Indeed, the inhibition of RA synthesis with inhibitors against ALDH1 enzymes decreased podocyte formation [76]. In support of these finding, a mild deficiency of vitamin A (RA precursor), which in other organs did not cause any significant abnormalities, in the kidney established a reduction of nephron number. Furthermore, a severe deficiency of vitamin A induced renal aplasia/hypoplasia and ureteral abnormalities [77].
- From a clinical point of view, several studies showed that RA protects against renal injury including minimal change disease, membranous nephropathy, FSGS, human immunodeficiency virus associated nephropathy (HIVAN) and lupus nephritis [78]. Consistently, in vitro RA represented the key inducer of human renal progenitor differentiation toward the podocyte lineage, allowing the acquisition of specific markers and features of podocytes. The administration of RA in vivo in experimental models of FSGS and membranous nephropathy determined an increased number of proliferating PECs lining the Bowmaṅs basement membrane and of podocytes. Notwithstanding RA treatment was associated with an increased number of cells with transitional phenotype, and no effect on proteinuria was observed [79]. A recent study explained these results showing that exposure to albumin can impair RPC differentiation into podocytes by sequestering RA. It is well known that albumin is the specific carrier of RA in the blood and binds it with high affinity. Thus, small amount of albumin present in Bowman space can sequester RA, impairing the activation of RARE-mediated transcription of podocyte-specific genes. For this reason, RPC differentiation toward podocytes cannot develop fully and the glomerular damage is not repaired [80]. In addition, inhibition of RA synthesis in a model of FSGS worsened

albuminuria and mortality suggesting that endogenous RA is essential for podocyte number maintenance. These results make the modulation of the RA-albumin balance as a target for therapeutic strategies in glomerular diseases. Drugs could operate either by reducing proteinuria, such as ACE-I that halts the progression of chronic kidney disease in patients with diabetic and nondiabetic nephropathies [81], or by increasing the progenitor's response to RA, for example, elevating the endogenous levels of RA. In this regard, a phase II clinical trial of isotretinoin-treated patients with FSGS and collapsing glomerulopathy, who were resistant to standard therapy, found no significant changes in proteinuria at week 24 compared with baseline levels (ClinicalTrials.gov identifier NCT0098020). However, only seven patients were enrolled, due to concerns regarding the adverse effects of retinoids, and the results are inconclusive. Recent results from Lasagni and colleagues [13] corroborated the hypothesis that pharmacological approaches that increase podocyte responsiveness to RA signaling would mitigate progression of experimental renal injury. Indeed, in vitro treatment of human RPCs with RA in presence of 6-bromo-indirubin-3′-oxime (BIO), a glycogen synthase kinases 3 (GSK3) inhibitor, induced a strong differentiation of human RPCs toward podocytes through activation of RARE transcriptional activity, that is, increasing RPC sensitivity to the differentiating effect of endogenous RA. Enhancement of RPC differentiation into podocytes by using BIO in a murine model of FSGS avoided toxicity and resulted in an important effect on the disease, shifting the outcome from chronicization to remission in all treated mice. BIO would be valuable alternative to RA and should represent a therapeutic strategy in glomerular diseases.

- *Dach1*. In 2010, through genome-wide association studies, researchers identified a link between the transcription factor, Dach1, renal function, and chronic kidney disease. Dach1 is part of Eya-Six-Hox-Pax regulatory network, which is essential for proper kidney development [82]. Recently, Endlich and colleagues suggested that Dach1 plays an essential role for PEC cell fate decision. Podocytes expressed high levels of Dach1 in vitro and in vivo, while PECs expressed very low levels. When PECs were induced to express Dach1 by transfection, they acquired podocyte phenotype increasing synaptopodin, and WT1 proteins, and at the same time downregulating Pax2 [83]. In support of these findings, recent publication showed a positive correlation between glomerular Dach1 expression levels and glomerular filtration rate in patients affected by glomerulopathy [84].
- *Interferons*. Collapsing nephropathy and pseudocrescents are hallmarks of viral glomerulopathies, such as HIV- and parvovirus-nephropathy. In these viral glomerulopathies, IFN-α and IFN-β not only trigger local inflammation inside the glomerulus but also act on PECs and podocytes, with IFN-α causing cell-cycle arrest and inhibiting the migration of PECs and both suppressing renal progenitor differentiation into podocytes in vitro. In vivo, in a model of Adriamycin nephropathy, injections of either IFN-α or IFN-β aggravated proteinuria and glomerulosclerosis. Interestingly, only IFN-β induced podocyte mitosis that lead to podocyte loss via mitotic catastrophe [85].
- *Renin-angiotensin-aldosterone system*. Inhibiting renin-angiotensin-aldosterone system (RAAS) using ACE inhibitors and Ang II type I receptor blockers (ARBs) is the current gold standard for renal diabetic and nondiabetic disease treatment. Indeed, experimental and clinical studies have demonstrated RAAS inhibitors prevent proteinuria, kidney fibrosis, and slow decline of renal function and thus play a protective role in both early and end stages of kidney disease. The mechanisms responsible for the therapeutic effects of these drugs, as well as their renal cellular targets, are multiple and have been largely studied both in patients and in animal models of human kidney disease [86]. Recent studies demonstrated that drugs already used in clinical practice might exert their beneficial effects also by promoting RPC differentiation into podocytes. Indeed, in a rat model of glomerular injury treatment with ACE-I limited PEC proliferation and the consequent formation of crescents and the progression toward glomerulosclerosis. Reestablishment of normal glomerular architecture by ACE inhibition was associated with reduced activation of progenitors and restoration of their distribution along Bowman capsule [87]. These data concur to indicate PECs as a novel cellular target of ACEi therapy. Ang II, via the AT1 receptor, also contributes to proliferation of progenitor cell of Bowman capsule. Ang II receptor AT1 seems involved in the mechanisms responsible for RPC proliferation. AT1 expression is limited to rare CD24 + PECs in normal human kidney or in patients with membranous and diabetic nephropathies, but local production of Ang II induces strong upregulation on progenitor cells in the area of hyperplastic lesions in both rats and human proliferative diseases [88]. The contribution of the Ang II/AT1 receptor pathway to the abnormal behavior of renal progenitors in proliferative diseases is explained by experimental data demonstrating that ACEi therapy limits progenitor cell proliferation by restoring AT1 receptor expression. These results provide another explanation to the beneficial effects observed after ARB treatment. Indeed, ACE-I therapy given to a patient affected by crescentic glomerulonephritis restricted progenitor cell proliferation and normalized AT1 receptor expression on RPCs, associated concomitantly with the regression of hyperplastic lesions [88].

ARB medication attenuates also experimental mesangial proliferative glomerulonephritis (MsPGN) by modulating the differentiation of renal progenitor cells toward podocytes [89], but the mechanisms that regulate this effect remained unproven.

– *Leptin*. Reversal of diabetic nephropathy (DN) has been achieved in humans and mice, but only rarely and under special circumstances. As progression of DN is related to podocyte loss, reversal of DN requires restoration of podocytes. Observation on biopsies of patients affected by DN supported the possibility of cell cycle activation of progenitors located on Bowman capsule in response to podocyte loss, expression of podocyte and PEC markers and migration onto the glomerular tuft [90]. These findings are consistent with preclinical studies in the BTBR *ob/ob* murine model of reversible DN. Indeed, in this model Pichaiwong et al. described that leptin replacement results in near-complete reversal of both structural and functional parameters of DN [91]. In particular, leptin treatment resulted in a significant increase in podocyte density and number and in an increase of WT1-positive proliferating PECs. These findings suggest that PECs may be a potential reservoir to restore lost podocytes and that the differentiative capacity of PECs may be a key element for the regression of DN and its pharmacological modulation. The mechanism underlying this process was further described in a follow-up paper where they show that treatment of leptin-deficient *ob/ob* mice with a selective antagonist of the endothelin-1 type A receptor (ETAR) in combination with RAAS inhibition lead to an improved phenotype [92]. In particular, the authors observed a significantly increased glomerular podocyte number compared with controls, suggesting that PECs were a source for podocyte restoration.
– *Mechanical forces*. In the last few years, several publications have shown that the mechanical stimuli play an important role in the stem cell behavior, regulating maintenance of their undifferentiated state and supporting specific fate decisions [93, 94]. In particular, recent evidence demonstrated that substrate stiffness modifications, both in vitro and in vivo, could influence stem cell activity during the development, in tissue homeostasis and in disease course. We recently evaluated in vitro the effect of substrate stiffness modulation on CD133 + CD24 + RPC decision to proliferate and to differentiate. We demonstrated that RPC proliferation and migration increased as stiffness increased, while RPC capacity to acquire podocyte markers was modulated only in a narrow range of stiffness. Successively using chemical and genetic inhibitors, we demonstrated that Rho kinase activity is the biochemical signal involved in the CD133 + CD24 + RPC response to biomechanical stimuli [95].

Conclusion

A vast body of literature has shown that the parietal epithelium of Bowman capsule represents a reservoir of renal progenitors in adult kidney deputed to replacement and regeneration of podocytes, as well as of proximal tubular cells and in vitro and in vivo studies allowed discovering the complexity of molecular processes that regulate renal progenitors in physiology and in diseases. The first main outcome of this knowledge is that encourage regeneration and promote functional repair of glomerular injury, and even prevention and treatment of glomerulosclerosis may be possible. In addition, the discovery that renal progenitors of Bowman capsule can not only regenerate podocytes but also cause hyperplastic glomerular lesions in crescentic glomerulonephritis, collapsing glomerulopathy, FSGS, or the tip lesion, suggests that glomerular disorders may at least in part result from abnormal or inefficient regenerative responses to podocyte injury.

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CHAPTER

24

Reconstitution of the kidney glomerular capillary wall

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Introduction

The glomerulus is the network of capillaries within the kidney that function to filter the circulating blood and serves as the primary site for blood filtration [1, 2]. The glomerular filtration barrier (GFB) consists of a thin layer of glomerular basement membrane (GBM), the terminally differentiated epithelial cells known as the podocytes, and fenestrated glomerular endothelial cells. The GFB facilitates the selective removal of toxins and waste from blood [3, 4]. Additionally, there is mounting evidence that dysfunction or loss of podocytes contribute significantly to the initiation and progression of kidney disease, leading to proteinuria and nephron degeneration [5, 6]. The podocytes are essential for the integrity of the kidney's filter [5], and their positioning in the kidney makes them integral components of the glomerulus [7]. Reduced glomerular filtration rate and loss of functional glomeruli and podocytes are associated with the progression of chronic kidney disease (CKD) [4], which can result from several factors including metabolic abnormalities, hypertension, autoimmunity, and genetic predisposition. Worldwide, more than 10% of adults suffer from CKD which is considered a public health problem due to growing incidence [4, 8]. Even with multidrug therapy, progression to end stage kidney disease (ESKD) is often irreversible, and patients will eventually require maintenance dialysis or kidney transplantation. However, dialysis is not considered an ideal treatment plan due to its high cost and adverse impact on the patient's quality of life [8]. Transplantation therapy also has limitations that include severe shortage of transplantable organs as well as organ rejection [8]. It is therefore important to explore new therapeutic options to help improve patient outcomes. This goal gave rise to the prospect of utilizing stem cells to model human tissue, which together will serve as a therapeutic avenue.

Stem cells have significant proliferative and developmental capacity and, as such, possess great potential for advancing cellular therapy, disease modeling, and drug discovery [9]. Also, stem cells are highly advantageous as they can be differentiated into specialized kidney cells [10] or organoids [11, 12], which can be used to model kidney disease, and aid in drug discovery [13,14]. Despite recent progress in the stem cell field, it remains challenging to create organoids with functional characteristics as these structures typically produce cells with immature phenotype [15]. To better understand the mechanisms underlying kidney development, the pathophysiology of the glomerulus, and the underpinnings of glomerular disease, it is important to develop a more accurate model of the functional human kidney glomerulus. This need inspired recent developments in the field that aimed to model kidney and podocyte differentiation using mice [16], human embryonic stem cells (ES cells) [17,18], and human induced pluripotent stem cells (iPS cells) [19,20].

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We recently developed a protocol for generating functional kidney podocytes from human iPS cells using a novel chemically defined media. Our stem cell differentiation method yields podocytes with specificity and efficiency, providing an unlimited source of specialized kidney glomerular podocytes with characteristics comparable to the mature phenotype [10,21]. The modulation of a few signaling pathways that play an essential role in glomerular development enabled the rapid and efficient lineage conversion of human iPS cell derivatives into terminally differentiated cells that exhibit morphological, molecular, and functional characteristics of mature kidney glomerular podocytes. Furthermore, we used a microfluidic platform that facilitates the coculture of podocytes and endothelial cells in a dynamic tissue culture system that included fluid perfusion and cyclic mechanical strain to more closely model physiological conditions and drug responses [10].

In this chapter, we provide an overview of the development and physiology of the glomerulus and GFB. We discuss the features necessary for engineering functional in vitro model of the human kidney glomerulus that accurately reflects human physiology. We highlight our podocyte differentiation protocol, which yields functional and mature cell types of the human kidney glomerulus. Additionally, we detail the important characteristics of a glomerulus-on-a-chip device, including the configuration of the device (composed of biocompatible materials), the application of mechanical forces, filtration assessments, and disease modeling. Finally, the future outlooks of this technology are discussed, including applicability toward regenerative medicine and pharmaceutical screenings and beyond.

Structure and function of the glomerulus

The glomerulus is a tuft of capillaries lined by endothelial cells and encased by podocytes. The two cell layers are separated by a glomerular basement membrane. All three components function together to form the glomerular filtration barrier. The glomerulus receives blood through the afferent arterioles. Podocytes enable filtration of toxins and macromolecules from blood, and the filtered blood is transported back to the systemic circulation through efferent arterioles [22]. The glomerulus is a highly vascularized compartment of the human kidney that receives 1100 mL of blood per minute [23].

Overview of mammalian glomerulogenesis and the development of the glomerular filtration barrier

In embryology, the mammalian kidney develops in several stages, starting with the intermediate mesoderm, which undergoes anterior–posterior (AP) patterning guided by a delicate balance between retinoic acid and its inhibitors (Fgf8 and Cyp26) along the A-P axis [24–26]. The global variability of these factors divides the intermediate mesoderm into three distinct zones: (1) anterior intermediate mesoderm (high retinoic acid activity), (2) middle intermediate mesoderm (moderate Fgf8 activity), and (3) posterior intermediate mesoderm (high Fgf8 and Cyp26 activities) [26,27]. These morphogens provide chemotactic guidance to cells and drive mesenchymal-to-epithelial transition of the anterior intermediate mesoderm. Simultaneously, the pronephric duct extends posteriorly [28], and when it reaches the caudal end of the embryo, the ureteric bud invades the surrounding metanephric mesenchyme, marking the beginning of nephrogenesis (Fig. 24.1A). The ureteric bud then sprouts epithelial cells, a process mediated by glial -derived neurotrophic factor and retinoic acid secreted from the nephric duct into the metanephric mesenchyme [29]. At this time, Wnt9b emanating from the metanephros, with the help of BMP7, drives condensation of the metanephric mesenchyme to form the cap mesenchyme [28,29]. The cap mesenchyme is composed of a transit amplifying population or nephron progenitors at the tip (characterized by WNT11, BMP7, FGF9, and WNT9B), and nephron formation occurs at the stalk from the ureteric epithelium between the tip and the stalk [28,30–32]. The nephron progenitors express Wnt4, Fgf8, Pax8, and Lhx1 [33–35] and are clustered at the tip-stalk junction to form the pretubular aggregates (Fig. 24.1A). Further autocrine secretion of Wnt4 from pretubular aggregates drives the mesenchyme-to-epithelial transition, giving rise to cystic renal vesicles (Fig. 24.1A) (expressing polarity proteins aPKC and PAR3) [35,36].

The timing of the recruitment of nephron progenitor cells from the cap mesenchyme into the renal vesicle offers unique geometric and functional trajectories. Cells recruited in the early stages of development contribute to the formation of the distal tubular precursors (located closest to the ureteric bud tip), and the cells recruits later in the process form the proximal tubular precursors, and final recruits form the glomeruloid precursors. At this stage, structural changes to the cystic renal vesicle can be observed as it folds inwardly to form comma-shaped and S-shaped bodies [37]. These structural changes are accompanied by differential expression of Cadherin 1 (CDH1) from the proximal part closer to the ureteric epithelium and Jagged canonical notch ligand-1 (JAG1) from the medial domain. Simultaneously, a Wilms Tumor 1 (WT1) gradient is generated in the distal end of the S-shaped bodies away from

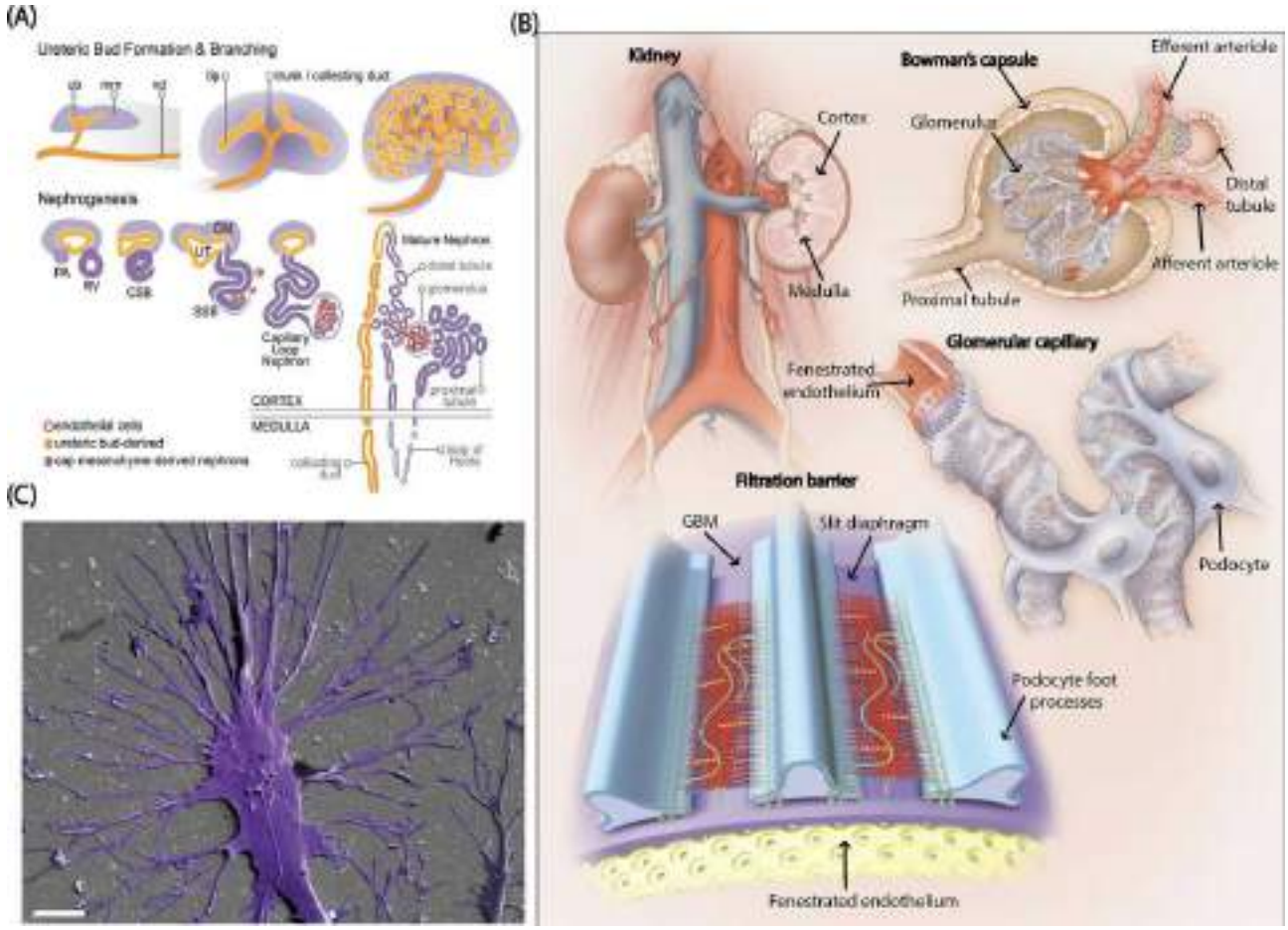

FIG. 24.1 (A) Overview of mammalian nephrogenesis. *CSB*, comma shaped body; *DT*, distal tubule; *Ub*, ureteric bud; *LoH*, loop of Henle's; *mm*, metanephric mesenchyme; *nd*, nephric duct; *PA*, peritubular aggregates; *PT*, proximal tubule; *RV*, renal vesicles; *SSB*, S-shaped bodies. Image reproduced with permission from The Quantified Kidney: A Human Frontiers Science Project Tutorials (B) Anatomy of human kidney. (C) Human iPS cell-derived podocytes. *Figure reproduced with permission from Musah S, Dimitrakakis N, Camacho DM, Church GM, Ingber DE. Directed differentiation of human induced pluripotent stem cells into mature kidney podocytes and establishment of a glomerulus chip. Nat Protoc 2018;13:1662–85 and Tryggvason K, Patrakka J, Wartiovaara J. Hereditary proteinuria syndromes and mechanisms of proteinuria. N Engl J Med 2006;354(13):1387–401. https://doi.org/10.1056/NEJMra052131.*

the ureteric epithelium that marks the beginning of glomerulogenesis when a single capillary invades the glomerular cleft [37]. This capillary then splits into six or seven capillary loops, thus delineating the capillary loop stage of nephrogenesis (Fig. 24.1A) [38].

The early glomerulus is composed of columnar epithelial cells (podocytes), a thin layer of squamous-like epithelial cells (Bowman's capsule), and capillary loops. During development, the immature podocytes and the endothelial cells secrete their own basal lamina expressing Laminin 1 α1β1γ1 and Type IV collagen α1 and α2. With time, these basal laminae fuse to form the nascent glomerular basement membrane, marked by the expression of Laminin 8 α4β1γ1. The fusion of basal laminae brings the two cell populations close to each other. Upon maturation of the glomerular basement membrane in S-shaped bodies, a second shift occurs in the expression of laminin, which switches from Laminin 8 α4β1γ1 to Laminin 10 α5β1γ1. The podocytes slowly start to lose their lateral cell attachment and extend their cell bodies around the capillary loops and begin to express significant levels of Laminin 9 α4β1γ1 and Laminin 8 α5β2γ1. Around this stage, the podocytes begin to extend their arborized network and foot processes, key structural features of podocytes that enable their filtration function Fig. 24.1B. This arborization ensures interdigitated packing to enwrap the glomerular capillaries, a process that is facilitated by the binding of α3β1 integrins and α-dystroglycans from the podocytes to Laminin 11 α5β2γ1 on the GBM [39,40]. Podocytes also promote the proliferation and maintenance of fenestrated endothelial cells via the secretion of pro-angiogenic molecules, including vascular endothelial growth factor (VEGFA), Angiopoietin 1 (ANGPT1), and Stromal cell derived factor 1 (SDF1) [41,42]. As such, these arborized podocyte networks with fenestrated endothelial cells are the key players of the GFB, and

the mature GBM at its final stage consist of several proteins including Laminin 11 α5β2γ1 and collagen type IV α3, α4, and α5 heterotrimers. The embryogenic decision check points described here, in combination with ECM matrix cues, can be employed to undergo stepwise differentiation of human stem cells to mimic the human kidney glomerulus (discussed in Section "A novel method for the directed differentiation of podocytes from human pluripotent stem cells" below) [43].

Glomerular basement membrane

The Glomerular Basement Membrane (GBM) is a thick, fibrous, and porous matrix that separates vascular endothelial cells from podocytes (Fig. 24.1B). In humans, the GBM is approximately 350 nm thick and is primarily composed of ECM proteins: Type IV collagen α3, α4, and α5 heterotrimeric chains, nidogen-1, 2, tubulointerstitial nephritis antigen like protein (TINAGL1), heparan sulfate glycoproteins (agrin, perlecan, collagen XVIII), and laminin 11 (α5β2γ1) synthesized by the podocytes and the endothelial cells [44]. The organization of the GBM involve concerted integration of receptors of varying polarity in relation to the local population of ECM proteins. Interestingly, the two most abundant molecules within the GBM, Laminin and Type IV collagen, are stabilized by numerous bridging molecules that form a polymeric network [45]. This network imparts size and charge selectivity, thereby minimizing the passage of anionic macromolecules. Below, we describe some of the different aspects of the ECM and GBM that function to maintain glomerular permselectivity.

The GBM serves is a major constituent of the kidney's blood filtration barrier, which prevents proteins or large molecules from being filtered into the urinary compartment as they travel through the glomerular capillaries. Within the GBM, the orientation of the α3α4α5 and α1α1α2 (IV) collagen networks are centralized, away from the surface of the podocytes, whereas laminin α5, agrin, and β1 integrins are aligned bimodally, toward the podocytes [46]. Agrin is the most prominent heparan sulfate proteoglycan of the GBM [47], and its N terminal binds with Laminin α5β2γ1, whereas the C terminal interacts with receptors such as integrins on podocytes, which together contributes to charge selectivity of the GBM [45,48]. Additionally, integrin clustering aids high-affinity adhesion of podocytes with the GBM. The principal integrin that aids podocyte adhesion to the GBM is integrin α3β1, which is further supported by tetraspanin CD151 [49]. Deficiencies in integrin α3 and CD151 can lead to a disorganized, laminated GBM and less specialized podocyte foot processes, resulting in severe proteinuria, focal segmental glomerulosclerosis (FSGS), and End Stage Kidney Disease (ESKD) [49–51]**.** In addition to integrins, dystroglycans confer adhesive cues to the podocytes by bridging actin filaments with laminin at the interface of podocyte foot processes and the GBM [52]. Anionic proteoglycans like syndecan-1 can also modulate adhesive strength of the podocytes by regulating integrin β3 expression through autocrine signaling [53]. The GBM is further characterized by focal adhesion kinases that are closely linked to integrins (via paxillin and talin), forming stabilized adhesomes to counteract GBM distention during filtration [54]. Wu and colleagues reported the formation of the ILK/PINCH/Parvin (IPP) complex (between PINCH, parvin, and kindlin-2 [55])—a pseudokinase in the center stage of cell-matrix adhesion and signaling that acts as a signaling hub for integrins and the podocyte actin cytoskeletal network. These linker proteins maintain the glomerular filtration barrier and control cytoskeletal dynamics, thus preventing foot process effacement and proteinuria [56–58].

The main gatekeepers of the glomerular filtration barrier are the podocytes and their slit diaphragms. Podocytes possess primary and secondary processes that wrap around the glomerular capillaries. These cells exhibit a dynamic cytoskeletal network that allows them to maintain intrinsic glomerular permselectivity, without clogging or fouling of the filter during molecular filtration. The arborized foot processes of the podocytes help them to physically adjoin with neighboring podocytes via interdigitations that results in formation of pores of approximately 12.1 nm [59]. The major determinants of the slit diaphragm include quasiperiodically spaced Nephrin like protein-1 and 3 NEPH1/3 and Nephrin (NPHS1), where Nephrin like protein-1 interacts in *cis* with NPHS1 and FAT1. NEPH1 [60,61], neural Wiskott-Aldrich syndrome protein (N-WASP), and actin related protein 2/3 (Arp2/3) [62] are critical proteins for arborized foot process development, whereas NPHS1 in the major slit diaphragm protein. NEPH1 is localized proximal to the GBM, and NPHS1 is found in the apical portion of the slit diaphragm. [63] NPHS1 contributes to cytoskeletal dynamics, inducing actin polymerization catalyzed by the degradation of N-WASP and p21-activated kinase (PAK) [64]. Takano, Holzman, and Terzi reported that NPHS1 can interact with the cytoplasmic target PI3K in a tyrosine phosphorylation-dependent manner to activate Akt [65] and cofilin [66], thereby helping with actin remodeling during mechanical stress [67]. The podocytes are enveloped by negatively charged agrin, perlecan, and other proteoglycans, along with podocalyxin, glypican-1, and syndecan-4 that contribute to charge selectivity of the slit diaphragm. The slit diaphragm is also rich in tight junction proteins, including ZO-1, CASK, spectrins, JAMA-A, cingulins, and IQGAP1 [68], which preserve the arborizations of the podocyte and prevent foot process fusion and

proteinuria with a selective charge barrier to proteins. Furthermore, it has been suggested that there is a direct link between podocyte adhesome and slit diaphragm signaling via the assembly of integrin-linked kinases with α-actinin 4. Additionally, the stomatin protein family member Podocin (NPHS2) localizes at the apical site of the slit diaphragm. NPHS2 expression is regulated by the activation and translocation of LIM homeobox transcription factor 1-ß (LMX1ß) into the nucleus [69]. The translocation of LMX1B generates a positive feedback loop for the expression of NPHS2 in response to mechanical/environmental stress to maintain structural plasticity. Mutations in LMX1B are associated with nail-patella syndrome, which affects the terminally differentiated phenotype of podocytes [70].

According to the Electrokinetic model proposed by Moeller and colleagues, an electrical field is generated along the glomerular capillary wall during filtration that helps migration of anionic molecules. The migration of molecules is guided by the repulsion from sialyzed residues, via electrophoresis [71]. In this process, NPHS1 regulates permeability of the GBM via compression in an increasing gradient from the lumen to the podocyte layer of the filtration barrier. In doing so, NPHS1 governs the size and charge selectivity of the glomerular filtration barrier. The electrophoretic anisotropy establishes a balance between radial compression and circumferential tension along the filtration barrier, favoring broad spatial dispersion of macromolecules and restricting transmission of proteins in the urinary compartment [72–74]. However, the mechanism of dynamic podocyte FP arborization in response to this electrochemical gradient of blood macromolecules remains unknown.

The local microenvironment modulates foot process arrangement and derangement cycles to accommodate high hydraulic permeability. For instance, the calcium channel TRPC6 helps podocytes to sense their microenvironment and supports cytoskeletal remodeling in response to glomerular pressure, fluid flow, and filtration rate [75]. Additionally, Phospholipase C can activate TRPC6, enabling a rapid influx of calcium ions into the podocytes [76]. As a result, high intracellular concentration of Ca(II) reduces NPHS1 and synaptopodin expression at the slit diaphragm, upregulating RhoA activity and actin derangement [77]. Another multifunctional scaffold protein, CD2 associated protein (CD2AP), binds to NPHS1, NPHS2, synaptopodin, and Src substrate cortactin and regulates actin nucleation via the Arp2/3 complex [78–81]. During injury, the podocytes undergo foot process effacement and retraction, which fail to counteract the high hydrostatic filtration pressure that leads to proteinuria. Further investigation is necessary to understand how these signaling molecules are disrupted during pathogenesis of proteinuria. Although most of the studies to date have focused on immortalized cell lines and animal models, emerging technologies in microfluidic systems can be developed using human iPS cell-derived tissues to mimic miniaturized humanized biological functions as described below.

Considerations for recapitulating the glomerular filtration barrier in vitro

The unique cellular microenvironment, the tissue-tissue interface, and the suite of molecular signals, among other factors described above, all contribute to the sophisticated filtration processes that occur within the kidney. Contributing to this complexity are the chemical signals and conditions (such as appropriately timed transcription factors, matrix mechanics at the interface, and mechanical forces associated with renal blood flow) that undergo changes during distinct stages of development. The delicate nature of the glomerular capillary wall, complexity of bottom-up tissue engineering strategies that aim to simulate tissue development and function, and lack of reliable podocyte cell source have made it particularly difficult to develop in vitro models of the kidney glomerulus in vitro. To overcome these limitations and to more accurately engineer the glomerular capillary wall, an in vitro platform should ideally conform to a set of criteria that closely mimic the dynamic structure and functional properties of the human glomerulus. These criteria include:

(1) Cells that exhibit phenotypic and functional characteristics of human podocytes as observed in vivo.
(2) A dual-chamber device for cell culture containing appropriate matrix mechanics and media for endothelial cells (vascular chamber) and podocytes (urinary filtrate chamber).
(3) A synthetic or naturally derived basement membrane separating the two compartments or cell layers (podocytes and endothelial cells), and capable of recapitulating selective molecular permeability and cellular crosstalk between the endothelium and epithelial cells.
(4) A device that supports fluid flow and mechanical force manipulations to simulate renal blood flow, pressure, and tissue stretching and relaxation motions as observed in glomerular capillaries in vivo.
(5) Biochemical testing capabilities including molecular filtration rates, cellular viability, and transcriptomics via chemical sampling and/or microscopic imaging within the device itself or effluents from microfluidic chambers.
(6) Compatibility with disease modeling (including drug-induced or generic forms of kidney glomerular diseases).

Engineered model of human kidney glomerulus

One of the primary goals of tissue engineering is to make available cells and tissues that can advance understanding of physiological processes, disease mechanisms, and facilitate therapeutic development including tissue replacement strategies. These advances hinge upon the ability to obtain reliable sources of functional human cells, which remains a significant challenge in nephrology, especially when the cells of interest (e.g., podocytes) develop their full phenotypic and functional characteristics during late stages of development [82]. Additionally, most methods for the differentiation of human iPS cells into organ-specific cell types yield cells with fetal-like characteristics [11,15], thus, limiting their utility when trying to model postnatal human physiology and pathophysiology. Thus, it is critical to develop methods to produce organ-specific cell types with adult-like functional properties. Glomerular podocytes are a prime example of an organ-specific cell type that is highly specialized at maturity. The mature cell has well-defined functions and a complex cytoarchitecture [83]. Mature and fully differentiated podocytes have limited proliferative capacity, [15,84] limiting the capacity to endogenously repair injury in the kidney glomerulus. Mechanisms of cellular repair in podocytes are extremely limited, as podocytes lack the necessary cell cycle machinery to replenish cell loss [85]. As a result, the loss or dysfunction of podocytes is often irreversible, and this loss can give rise to glomerular sclerosis, the leading cause of nephrotic syndrome in adults [85,86].

There are several established podocyte cell lines, but their functional capabilities often do not accurately reflect in vivo functionality. Conditionally immortalized human podocytes are widely utilized to study podocyte biology [87,88]. though they differ phenotypically and biochemically from podocytes in vivo, as evidenced by differences in the expression of podocyte markers, response to toxins, and motility [86]. Additionally, primary human podocytes are difficult to culture for many reasons, including the nonproliferative nature of these cells, their tendency to dedifferentiate, and sporadic effacement or loss of foot processes [19,86], which all make it difficult to obtain sufficient quantities of mature podocytes for basic research.

Human iPS cells provide an attractive cell source to generate mature podocytes, as they are easily derived (without the ethical controversy associated with embryonic stem cells [9]), are biocompatible, and can differentiate into nearly any cell type under appropriate conditions [89]. Furthermore, iPS cells can be used in place of embryonic stem (ES) cells as they resemble ES cells in gene expression, pluripotency, and epigenetic state and have the ability to self-renew indefinitely [90]. In this section, we will discuss methods to differentiate human iPS cells into mature kidney podocytes, including details on the cell culture conditions, established differentiation protocols, and the filtration and tissue-tissue interface capabilities of stem cell-derived podocytes. Several other differentiation protocols have been reported for the derivation of podocytes from stem cells [86,91,92]. However, we will focus our discussions below on a more recent protocol we previously reported for robust and specific differentiation of human iPS cells into mature and functional kidney glomerular podocytes [10].

Extracellular matrices for cell adhesion and differentiation

An effective and specific culture condition is essential to fully exploit the benefits of using human iPS cells for organ-specific tissue culture. Chemically well-defined substrates are often utilized to support human iPS cell self-renewal [93] and simulate cellular microenvironment, including the extracellular matrix (ECM) [94]. The ECM is a major component of the glomerular microenvironment, and it possesses distinct physical, biochemical, and biomechanical properties [95], and all three provide the cell with adhesive, structural, and mechanical signals. A variety of factors within the local tissue (such as cell–cell and cell-matrix interactions) influence specific cell fate decisions. The composition of the GBM is important in vivo, as it contributes significantly to glomerular development and disease processes [96]. When designing an in vitro model of biological systems, matrix mechanics can also be modeled or controlled in a way that synergizes with other factors to guide appropriate cell fate decisions [93]. It has been observed that soft biomaterials are unable to support cell propagation due to insufficient cell adhesion to the matrix, the impact of the actin cytoskeleton of cells, and poor cell–cell interactions [93]. Together, these factors ultimately lead to the detachment of stem cell colonies from the matrix. Stiffer matrices, however, were shown to promote stem cell adhesion and spreading. Thus, a key quality found to promote stem cell self-renewal is matrix stiffness, as stiff substrata are more effective for the propagation of human ES and iPS cells.

In optimizing conditions for human iPS cell differentiation to podocyte, we considered several ECM proteins, and ultimately selected laminin and its fragments for multiple reasons including presence of laminins in the GBM both during tissue development and maturation [83,97] We examined cell adhesion under serum-free conditions on surfaces coated with various ECM proteins such as laminin-511, laminin-511 E8 fragment, or laminin 521 and found that all these coatings promoted the attachment and propagation of dissociated human iPS cells which type

1 collagen was suboptimal for the attachment of human iPS cells [10]. Furthermore, surfaces functionalized with laminin-511, laminin-511 E8 fragment, or laminin 521 supported the ability of soluble inductive factors to promote adhesion of human iPS cells and the directed differentiation of iPS cells into the intermediate mesoderm lineage. We selected laminin-511 E8 fragment as the matrix in our differentiation protocol because laminin-511 E8 exhibited higher cell-binding efficiency at a lower cost than other options.

A novel method for the directed differentiation of podocytes from human pluripotent stem cells

Until recently, it has been difficult to direct pluripotent stem cell differentiation into podocytes with specificity and efficiency. Our differentiation method provides a simple and robust three-stage protocol for the differentiation of mature kidney podocytes from human iPS cells in chemically defined and feeder-free conditions. Our approach involved chemically defined soluble growth and signaling factors, coupled with the insoluble ECM matrix (discussed above) to promote the differentiation of human iPS cells into mature podocytes. Our differentiation method mimics three developmental steps important for podocyte development (Fig. 24.2A). During development, the glomerulus and nearly all kidney cells develop from the mesoderm germ layer, passing through an intermediate step called the intermediate mesoderm [98]. Thus, the three steps in our protocol give rise to three distinct cell types, coined by the stages of development they represent: (1) Mesoderm, (2) Intermediate Mesoderm, and (3) Podocyte (Fig. 24.2A). We began the mesoderm-induction step by seeding dissociated human iPS cells on a tissue-culture plate precoated with laminin-511 E8 fragment in a defined mesoderm-induction medium. The mesoderm differentiation medium contain activin A, CHIR99021 [99] and Rho-associated kinase (ROCK) inhibitor Y27632 which improves cell survival after iPS cell dissociation and reduces dissociation-induced apoptosis [100]. The cell culture media was changed daily, and, after 2 days in the mesoderm induction medium, the cells resembled the mesoderm germ layer, expressing the mesoderm markers, goosecoid, HAND1, and brachyury in almost all cells [10]. Then, to induce an intermediate mesoderm cell type, the stem cell-derived mesoderm cells were fed with intermediate mesoderm induction medium containing bone morphogenetic protein 7 (BMP7, an essential signaling molecule during kidney development [101]) and CHIR99021 for a minimum of 14 days to induce the intermediate mesoderm lineage. At day 16, cells expressed the intermediate mesoderm and metanephric nephron progenitor cell markers [92,99] odd-skipped related 1 (OSR1), Wilms tumor 1 (WT1), and PAX2. At the intermediate mesoderm stage, cells are still capable of proliferating, evidenced by the ability to incorporate EdU into DNA. Importantly, intermediate mesoderm cells can be maintained in culture for almost 60 days with passaging every 8–12 days or cryopreserved.

For the final step of our podocyte differentiation protocol, we developed a novel podocyte-inducing differentiation medium comprised of soluble factors that modulate key signaling pathways involved in glomerular development and podocyte lineage determination in vivo. Our podocyte induction media contains vascular endothelial growth factor (VEGF), retinoic acid, CHIR99021, BMP7, and activin A. The rationale for these choices are as follow: (i) genetic and immunological studies have shown that VEGF is essential for glomerular capillary growth and differentiation of podocytes [102–105]; (ii) retinoic acid has a profound effect on growth differentiation and development [106] and enhances the expression of podocyte-specific markers nephrin and WT1 [107–109]; (iii) CHIR99021 activates canonical Wnt signaling [92], accelerating the formation of glomerular structures from embryonic nephron progenitors; (iv) BMP7 promotes nephrogenic cell survival during kidney development [110]; and (v) activin A improves the expression of nephrogenic genes in animal cap tissue when used in conjunction with retinoic acid [106]. It is important to note here that our protocol purposefully only employs chemically defined media and omits the use of poorly defined animal serums that often compromise the reproducibility of tissue cultures. Within 4–5 days of treatment with our podocyte-inducing medium, morphological features displayed by glomerular podocytes were observed in cell cultures (Fig. 24.2B). These features included an arborized cell body with a prominent nucleus and multiple finger-like cell protrusions. The treatment yielded large cells that resemble podocytes obtained from human kidney biopsy (Fig. 24.2C and D). Biochemical analysis via flow cytometry revealed that differentiated cells expressed the podocyte-specific proteins nephrin and WT1, and there was a concomitant decrease in pluripotency marker Oct4 (Fig. 24.2E). This method for podocyte induction was remarkably efficient, as more than 90% of the human iPS cell-derived podocytes expressed both nephrin and WT1 (Fig. 24.2F). We confirmed that our differentiation method can be used on multiple human iPS cell lines (e.g., DU11, IMR-90-1, and IISH3i- CB6 human iPS cell lines) [10,21,111] resulting in cells with characteristics similar to those of mature kidney podocytes. The efficiency of our podocyte differentiation protocol surpasses that of most other widely employed methods that differentiate human iPS cells into specialized organ-specific cell types [82], which highlights the specificity and potency of the conditions we identified for podocyte differentiation in vitro.

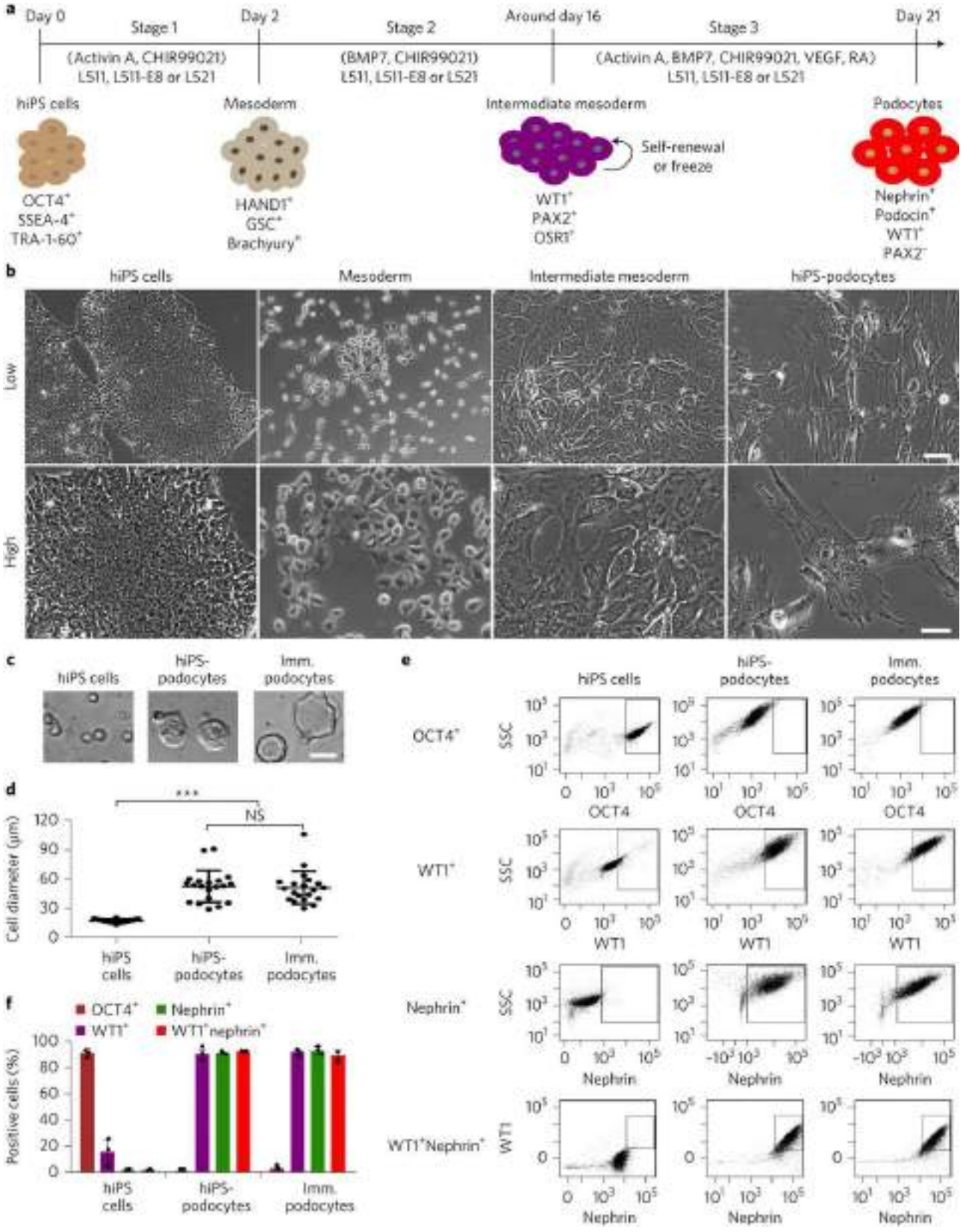

FIG. 24.2 Differentiation of human iPS cells into kidney podocytes. (A) Schematic outline for the directed differentiation of human iPS cells into podocytes. *VEGF*, vascular endothelial growth factor; *RA*, retinoic acid; *BMP7*, bone morphogenetic protein 7; *L511*, laminin-511; *L511-E8*, laminin-511-E8 fragment; *L521*, laminin-521. (B) Brightfield images of cells at low and high magnification at each stage of differentiation. *hiPS-podocytes*, human iPS cell-derived podocytes (C) Brightfield images showing the diameter of dissociated human iPS cells, human iPS cell-derived podocytes, and immortalized (Imm.) human podocytes. (D) Scatter plot of the diameter of dissociated human iPS cells, human iPS cell-derived podocytes, and immortalized (Imm.) human podocytes. (E) The expression of pluripotency and podocyte markers in human iPS cells, human iPS cell-derived podocytes, and immortalized (Imm.) human podocytes quantified by flow cytometry analysis. The representative plots show expression levels of OCT4 (a pluripotency marker), WT1 (kidney cell marker), and Nephrin (podocyte-specific marker) as well as dual expression of WT1 and nephrin. (F) Quantitative representation of the flow cytometry analysis showing percentage of cells positive for OCT4 (*brown*), WT1 (*purple*), and nephrin (*green*) and double positive for WT1 and nephrin (*red*). Scale bars, 100 μm (B, *top*) and 50 μm (B, *bottom*; C). Error bars show mean ± s.d.; $n = 20$ cells (D) or $n = 3$ independent experiments (F); ***$P < 0.0001$; *NS*, not significant. *Figure reproduced with permission from Musah S, et al. Mature induced-pluripotent-stem-cell-derived human podocytes reconstitute kidney glomerular-capillary-wall function on a chip. Nat Biomed Eng 2017;1:1–12.*

Gene analysis for pluripotency and podocyte markers (POUF1, PAX2, WT1, NPHS1, and NPHS2) revealed comparable expression levels in the human iPS cell-derived podocytes and the immortalized cell line with the exception of NPHS2 encoding podocin, for which higher expression was observed in the human iPS cell-derived podocytes. Genes typically expressed in progenitor cells, such as PAX2, were significantly downregulated in the human iPS cell-derived podocytes when compared to the immortalized podocyte cells. Gene expression for NPHS1 encoding nephrin was high, but low levels of the nephrin protein were observed, which is consistent with other findings [16,91]. Furthermore, a multifaceted approach using immunofluorescence analysis (Fig. 24.3A and B), flow cytometry, and gene expression confirmed higher expression of nephrin, WT1, and podocin in the human iPS cell-derived podocytes, accompanied by the absence of pluripotency proteins, OSR1, and PAX2 proteins. In our human iPS cell-derived podocytes, nephrin was more localized to the cytoplasm compared to the immortalized podocytes, which exhibited predominantly nuclear staining (Fig. 24.3C). Thus, the subcellular localization of nephrin in our stem cell-derived cells is consistent with the development of more mature podocyte phenotype [112,113].

We also confirmed that human iPS cell-derived podocytes were terminally differentiated, revealed by their inability to incorporate EdU. Together, these results indicate that our human iPS cell-derived podocytes are developmentally more mature than immortalized podocyte cell lines and resemble in vivo podocytes within functional glomeruli. We established an innovative method based exclusively on chemically defined factors and have shown that our differentiation media is capable of inducing podocyte differentiation with high reproducibility and offers an improvement over other protocols by generating more mature podocytes. We also demonstrate in Section "Engineering a glomerulus-on-a-chip that reconstitutes human kidney glomerular function in vitro" that our human iPS cell-derived podocytes can be cocultured with human kidney endothelium in an organ-on-a-chip microfluidic device, which underscores the portability of our protocol into different biomaterial platforms and offering novel microphysiological systems to investigate kidney glomerular function, drug toxicities, and mechanisms of disease.

The podocyte foot processes are elongated interdigitating structures that also interact with the GBM, forming a zipper-like structure [2,114,115]. These cellular structures contribute to the function of the GFB [104]. Immunofluorescence analysis of our human iPS cell-derived podocytes revealed bright podocin staining in both the cell bodies and the narrow membrane protrusions, a feature lacking or significantly underdeveloped in the established immortalized podocyte cell line (Fig. 24.4A). The presence of both primary and secondary processes in the cells was further confirmed by scanning electron microscopy analysis (Fig. 24.4A). These findings are consistent with previous reports indicating that immortalized podocytes do not form well-defined foot processes [5,87,104]. These data also demonstrate that our human iPS cell-derived podocytes have structural features similar to those of kidney glomerular podocytes in vivo [83].

Podocytes also play an essential role in maintaining the glomerular filtration barrier by enabling selective molecular filtration or the ability to filter molecules based on their size. [83] Podocytes do this by wrapping their interdigitated foot processes around the glomerular capillaries. At the apical side, podocytes are exposed to the primary urine, and at the basal cell membrane domain, they encase portions of their cellular processes into the GBM [83] via integrins and dystroglycans [20]. The integrity of the foot process is vital for the establishment of stable cell–cell and the cell-matrix interactions [81]. Foot processes along the GBM are connected through intercellular junctions, or slit diaphragms, which facilitate the passing of primary urinary filtrate but not high-molecular-weight plasma protein [81,112]. The filtration barrier is freely permeable to water and small solutes, and the slit diaphragm of podocytes is responsible for the size selectivity of the filtration barrier. Albumin, for instance, is a high molecular weight protein abundant in human plasma that is retained in the bloodstream under normal conditions. Thus, loss of albumin into the urine is a sign of kidney dysfunction, and the severity of glomerular injury in mice and humans can be assessed by the level of albumin in urine (additional information on glomerular filtration measurements is provided in Section "Testing of filtration function in an engineered model of the glomerulus").

Using our podocyte differentiation method, we observed evidence of the signature silt diaphragms present in podocytes and identified aspects of filtration functionality. Protein kinase C lambda/iota (PKCλ/ι), a cell polarity regulator maintaining the integrity of the glomerular slit diaphragm through nephrin trafficking to the cell surface [114], was identified in both nucleus and cytoplasm in the human iPS cell-derived podocytes via immunofluorescence staining and western blot analysis [10]. Western blot analysis revealed increased phosphorylation of PKCλ/ι (Fig. 24.3D and E), and immunofluorescent microscopy showed increased deposit of basement membrane collagen type IV. Both human iPS cell-derived podocytes and immortalized podocytes express the putative neonatal Fc receptor (FcRn) (Fig. 24.4B), which mediates the sequestering of large molecules in the glomerulus [102]. Additionally, the human iPS cell-derived podocytes showed greater ability than immortalized podocytes to take up exogenous albumin (Fig. 24.4C and D), a key process in glomerular filtration that is made possible by increased expression of FcRn. Thus, these results confirm that our differentiation method efficiently led to the production of human iPS

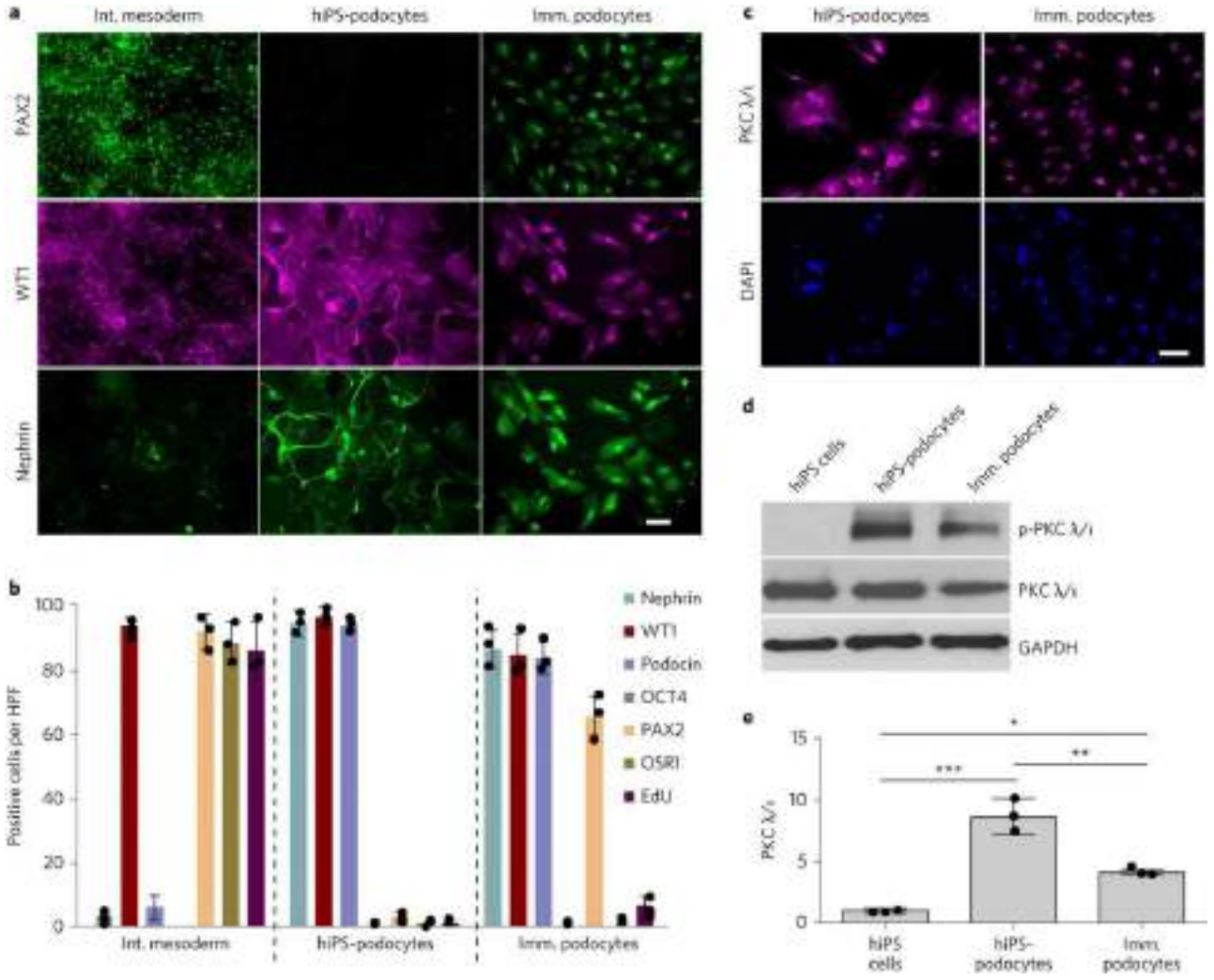

FIG. 24.3 Human iPS cell-derived podocytes express markers associated with the mature phenotype. (A) Immunofluorescence microscopy images of intermediate (Int.) mesoderm cells, human iPS cell-derived podocytes, and human immortalized (Imm.) podocytes stained for PAX2, WT1, and nephrin. (B) Cell number quantification of human iPS cell-derived podocytes showing upregulation of podocyte markers (nephrin, WT1, and podocin) and a decrease in the pluripotency marker OCT4. HPF, high-power field; EdU, 5-ethynyl-2′-deoxyuridine; OSR1, odd-skipped related transcription factor protein 1. The human iPS cell-derived podocytes showed a decrease in progenitor cell markers PAX2 and OSR1 and inability to incorporate EdU, indicating that these cells are postmitotic and terminally differentiated, as expected for mature podocytes. (C) Fluorescence microscopy images of human iPS cell-derived podocytes and immortalized podocytes stained for PKCλ/ι, a nephrin trafficker to the cell surface. (D) Western blot analysis of total and phosphorylated (p-) PKCλ/ι protein levels in human iPS cells, human iPS cell-derived podocytes, and human immortalized podocytes. GAPDH was used as loading control. (E) Quantification of phosphorylated PKCλ/ι levels obtained from western blot. Scale bars, 100 μm. Data are mean ± s.d.; $n = 3$ (B, E); $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$. *Figure reproduced with permission from Musah S, et al. Mature induced-pluripotent-stem-cell-derived human podocytes reconstitute kidney glomerular-capillary-wall function on a chip. Nat Biomed Eng 2017;1:1–12.*

cell-derived podocytes with morphological and molecular characteristics, and some filtration functionalities, consistent with mature kidney glomerular podocytes [10].

On the other side of the GBM are kidney endothelial cells that migrate to the podocyte tuft late during podocyte maturation in vivo [101]. Although the kidney endothelial cells were not required for podocyte lineage determination using our in vitro stem cell differentiation method, they do play an important role in podocyte function and establishment of tissue-tissue interface necessary for blood filtration and thus making the endothelial cells important for maintenance, survival, and function of podocytes [102]. Furthermore, there is crosstalk between the podocytes and glomerular endothelial cells in vivo, which is enabled by the exchange of soluble factors [101]. Organ-on-a-chip technology offers unique opportunities to mimic the GBM interface by coculturing podocytes and endothelial cells on a microfluidic cell culture platform. By recapitulating the cell–cell interface and applying mechanical forces to simulate renal blood flow, we showed that the resulting tissue model acquire high degree of cellular maturity and

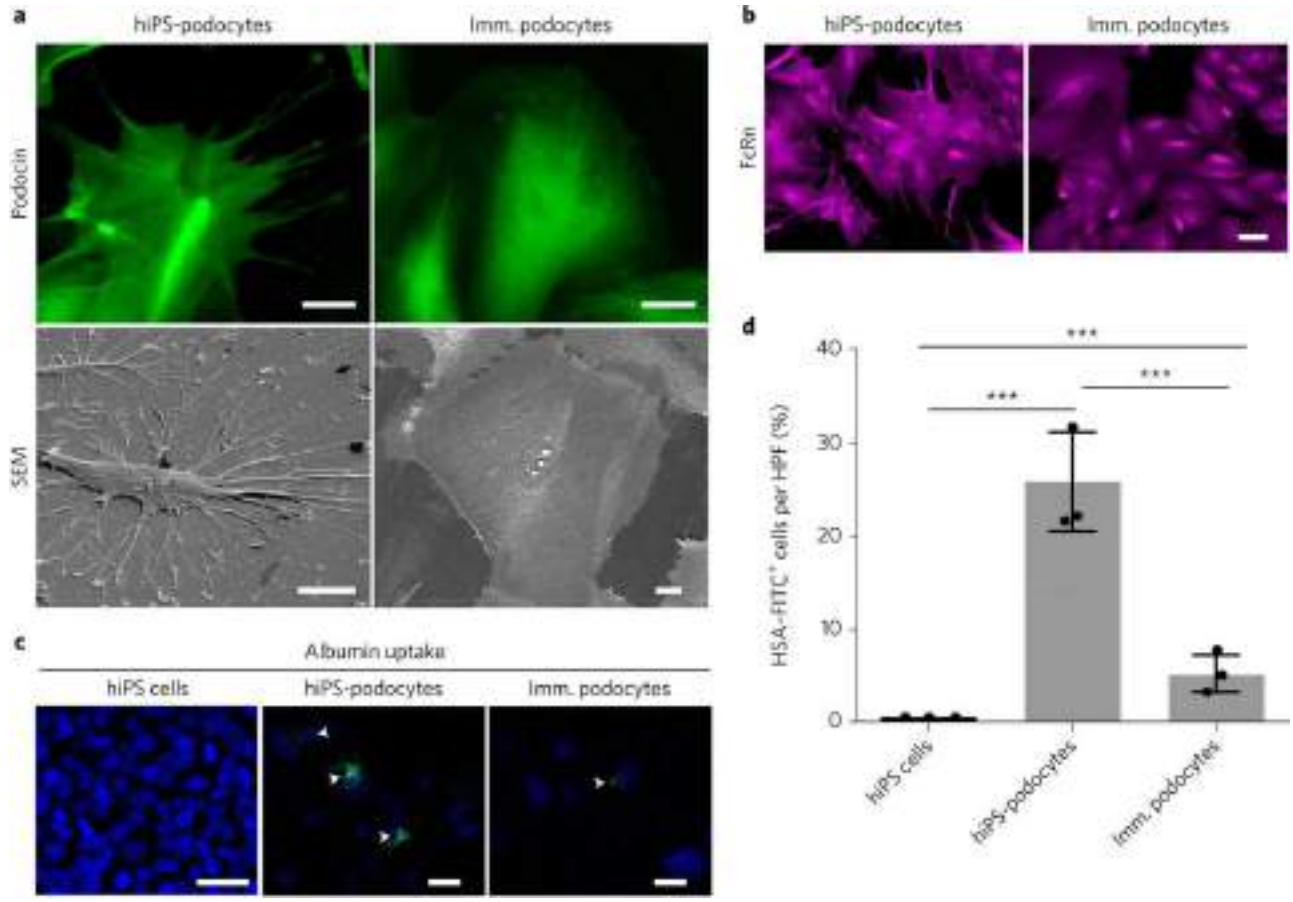

FIG. 24.4 Human iPS cell-derived podocytes exhibit primary and secondary cell processes and an ability to endocytose exogenous albumin. (A) Top panel, immunofluorescence microscopy images of human iPS cell-derived podocytes and human immortalized (Imm.) podocytes stained for podocin (*green*). *Bottom* panel, scanning electron microscopy (SEM) images of human iPS cell-derived podocytes and human immortalized (Imm.) podocytes. (B) Staining for FcRn (magenta), a receptor for albumin and IgG transport, in human iPS cell-derived podocytes and human immortalized (Imm.) podocytes. (C) Confocal microscopy images showing human iPS cells, human iPS cell-derived podocytes, and human immortalized (Imm.) podocytes exposed to exogenous albumin (HSA-FITC, *green*). Arrowhead pointing to cells that display albumin uptake. (D) HSA-FITC quantification of cells from (C). Scale bar, 10 μm (A, *bottom*), 50 μm (A, *top*; C) and 100 μm (B). Data are mean ± s.d.; $n = 3$; ***$P < 0.0001$. *Figure reproduced with permission from Musah S, et al. Mature induced-pluripotent-stem-cell-derived human podocytes reconstitute kidney glomerular-capillary-wall function on a chip. Nat Biomed Eng 2017;1:1–12.*

exhibit improved functionality over isolated cultures. We discuss our work, and the work of others, toward designing a model of the human glomerulus on-chip in the following section.

Engineering a glomerulus-on-a-chip that reconstitutes human kidney glomerular function in vitro

In designing an in vitro model of the glomerular filtration barrier, several conditions must be considered including the selection of synthetic GBM that supports propagation of the podocyte-GBM-endothelial tissue interface and discrete cell-ECM interactions. Additionally, mechanical forces, such as mechanical strain and fluid flow sheer stress (FFSS), must be reconstituted in a cyclic fashion because both the blood and urinary compartments of the glomerulus experience high hydraulic pressures governed by the dynamic pulsatile nature of blood and urine flow. Microfluidic organ-on-a-chip culture models offer a platform for mimicking the functional units of the kidney [115,116]. These biomimetic microsystems possess the structural features and cytoarchitecture of the organ-specific cell types that they are modeling, and the configuration of the substrate serves to reconstruct the chemical and mechanical microenvironment found in vivo. However, until the development of a protocol for deriving mature and functional human podocytes from human iPS cells as described in Section "A novel method for the directed differentiation of podocytes from human pluripotent stem cells," establishment of a glomerular tissue culture model remained a significant challenge. Thus, in this section, we will describe the recently described microengineered device coupled with

podocyte and endothelial cell cocultures that was designed to recapitulate the structure and function of the human kidney glomerulus [10]. Based on the criteria described in Section "Considerations for recapitulating the glomerular filtration barrier in vitro," significant emphasis will be placed on the mechanochemical features, functional characterizations, and capabilities for sample analysis that will be required for an in vitro model of glomerular function and disease.

Design of microfluidic organ-on-a-chip devices

The configuration of the microengineered fluidic device is important for reconstituting the filtration processes that occur at the glomerular capillary wall. Fig. 24.5A provides a schematic representation of the glomerular capillary wall or filtration barrier, consisting of a semipermeable layer formed by endothelial cells, podocytes, and the GBM. To simulate glomerular filtration, we microfabricated a glomerulus-on-a-chip device that possess two fluidic chambers with

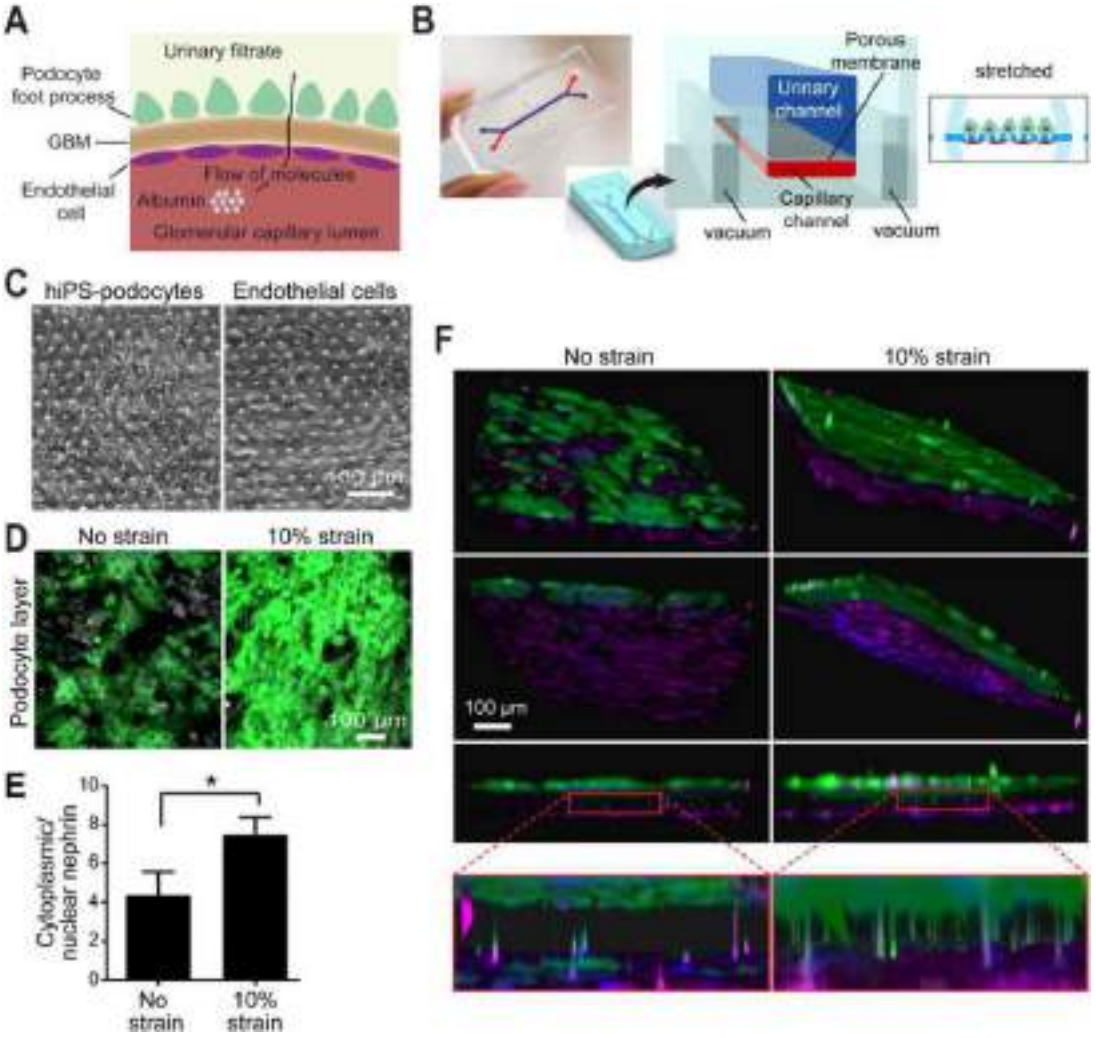

FIG. 24.5 A microfluidic organ-on-chip device that mimics the glomerular capillary wall. (A) Schematic illustration of the glomerular capillary wall. (B) Photograph and diagram of microfluidic organ-on-a-chip device containing vacuum channels for applying mechanical stretch and relaxation motion of the tissue layers. (C) Brightfield images of human iPS cell-derived podocytes and endothelial cells on the microfluidic chip. (D) Fluorescence microscopy images of human iPS cell-derived podocytes differentiated with or without 10% mechanical strain. Cells were immunostained for nephrin (*green*) and counterstained with DAPI (*blue*). Scale bar, 100 μm. (E) Quantification of cytoplasmic to nuclear nephrin under the presence and absence of 10% cyclic mechanical strain (error bars denote standard deviation of the mean, $n = 3$, $^*P < 0.05$.). (F) 3D reconstructed images of the podocyte-endothelial cell interface (podocytes in *green*, and glomerular endothelial cells in magenta) with and without 10% cyclic mechanical strain. Scale bars, 100 μm. *Figure reproduced with permission from Musah S, et al. Mature induced-pluripotent-stem-cell-derived human podocytes reconstitute kidney glomerular-capillary-wall function on a chip. Nat Biomed Eng 2017;1:1–12.*

one chamber representing the vascular compartment lined with endothelial cells, and the other represents the urinary compartment lined with podocytes (Fig. 24.5B). The two-cell layers are separated and interfaced by an engineered GBM.

We [10] and others [117,118] have utilized flexible poly(dimethylsiloxane) (PDMS) elastomer for crafting the multichannel cell culture substrate within our glomerulus-on-a-chip device. PDMS offers several advantages over other synthetic materials with regards to low cost, ease of fabrication, transparency (i.e., ease of coupling with optical measurement techniques), permeability to gasses (CO_2, O_2, etc.), and biocompatability [119]. Additionally, we functionalize the PDMS membrane with laminin 511 to serve as a scaffold for podocyte and endothelial cells adhesion and propagation [10]. Although others have employed Matrigel [117] or electrospun collagen nanofibers [120] or other types of materials for engineering organ-on-a-chip devices [4], we found that laminin-functionalized PDMS supported adhesion of iPS cell-derived intermediate mesoderm cells and their subsequent differentiation into podocytes.

The tissue-tissue interface is recreated by seeding endothelial cells in one channel and podocytes in the other. Podocytes were differentiated in situ in the microfluidic device from human iPS cell-derived intermediate mesoderm cells by using a chemically defined differentiation media (described above) in the urinary channel and endothelial cells were cultured in the vascular channel [10]. Brightfield images of differentiated podocytes and endothelial cells are shown in Fig. 24.5C—the podocyte induction protocol is described in Section "A novel method for the directed differentiation of podocytes from human pluripotent stem cells." By using this protocol in our microfabricated device (Fig. 24.5B), cells remained viable in culture for more than 2 weeks of propagation. Together, these methodologies (stem cell differentiation and microengineering of organ-on-a-chip devices) enabled recapitulation of the podocyte-GBM-endothelial interface, mimicking the urinary and vascular compartments of the human glomerulus.

Our model, along with the majority of organ-on-a-chip devices (both glomerular and other tissue models) [121], utilizes a planar scaffold [10]. It is conceivable that as the technology advances in the future, scaffolds with cylindrical shape could also be employed for modeling glomerular capillaries. Such technological advances remain challenging due to technical difficulties in synthesizing materials with complex structures without compromising their structural and functional integrity. We have found that, when applying methods on-chip that simulate the mechanical forces associated with vascular blood flow, our design is capable of recapitulating the human glomerular structure and function and allows for podocytes derived from human iPS cells to reach a higher level of maturity (evidenced by the downregulation of Pax2 and lack of EdU incorporation into DNA). We will discuss how the implementation of mechanical forces on the cellular models impacts maturity and differentiation efficiency on-chip in the next section. Additionally, more information on the design of microfluidic devices simulating the glomerulus and tubules can be found in the next chapter.

Role of mechanical strain on tissue development and function

A defining feature of the glomerulus and podocytes is the ability to thrive in an environment that experiences the mechanical forces associated with renal blood flow. Within the glomerulus, the flow of ultrafiltrate exposes podocytes to tensile stress, or stretch, as a result of pressure on the capillary walls. Interestingly, the mechanical forces placed on podocytes from the cyclic convection of blood flow is essential to their development and growth [122,123]. Thus, simulating the cyclic pattern of blood pressure and flow must be considered to maximize efficiency and maturity in an in vitro model containing glomerular podocytes.

Our engineered model of the kidney glomerulus offers advantages such as ease of fabrication and compatibility with in situ tissue analyses. To simulate the stretch and relaxation motions of glomerular tissues in vivo, we introduced dynamic mechanical stress typically resulting from cyclic pulsations of blood flow in living tissue. Ingber and colleagues first applied cyclic strain within a chip microenvironment in 2010 to recapitulate organ-level lung function [124]. By repurposing this technology to another organ system that is highly dependent on dynamic mechanical stress, we designed a planar model of the glomerulus in which we differentiated human iPS cell-derived podocytes under physiologically relevant mechanical conditions, using both fluid flow shear stress (FFSS) (0.0007 and 0.017 dyne/cm^2 in the urinary and vascular channels, respectively) and 10% cyclic strain (1 Hz, ~ 85 kPa) [122,123].

After 8 days of differentiation in the microfluidic device, the podocytes differentiated under mechanical strain expressed significantly more nephrin ($P < 0.0001$) than those in which mechanical forces were omitted (Fig. 24.5D). Podocytes in the top channel exhibited a significant increase in the expression of total nephrin ($P < 0.0001$) and a higher ratio of cytoplasmic to nuclear nephrin ($P < 0.05$) when exposed to mechanical strain (Fig. 24.5E). Podocytes differentiated under mechanical strain also secreted vascular endothelial growth factor (VEGF-A), as shown in Fig. 24.6A, an important signaling molecule for vascular patterning and glomerular development [125]. Together, these expression and secretion patterns are reflective of a higher level of podocyte maturation when exposed to

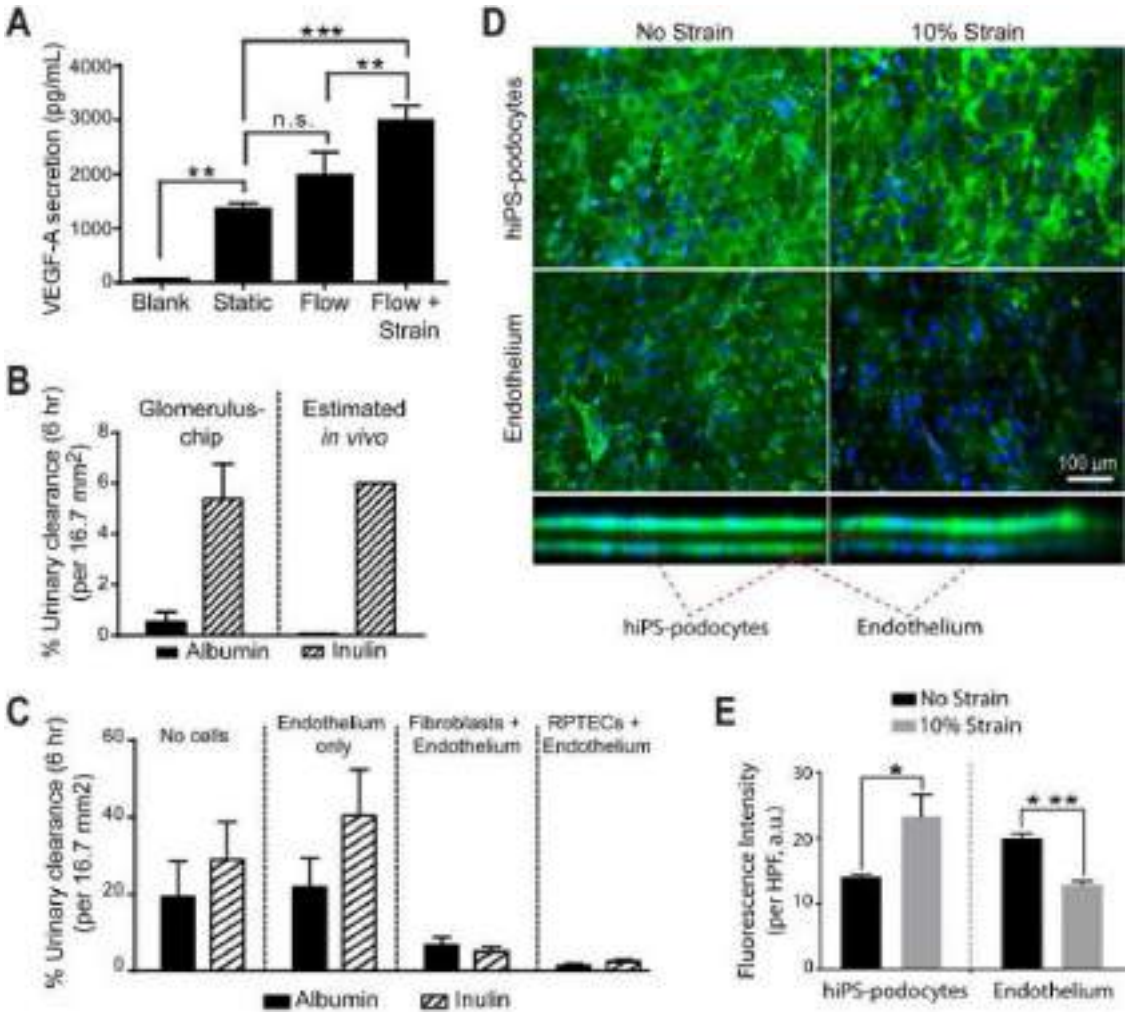

FIG. 24.6 Kidney glomerular function is recapitulated on an organ-on-a-chip device. (A) VEGF-A secretion by human iPS cell-derived podocytes on-chip. (B) Urinary clearance assessment via filtration efficiency measurements of albumin and inulin molecules over 6 h of infusion both on the glomerulus-chip and in vivo (estimated by the surface area of glomerular capillaries in vivo). (C) Filtration efficiency measurements of albumin and inulin over 6 h of infusion, repeated for glomerulus-chips in which podocytes were substituted by different cell types, demonstrating the cellular specificity of our model of the kidney glomerulus. (D) Immunofluorescence microscopy images of production and distribution of collagen IV (*green*) in the presence or absence of 10% cyclic mechanical strain on-chip (counterstained with DAPI, *blue*). Lower panel shows side view of chip. Scale bar, 100 µm. (E) Quantification of collagen IV in podocyte and endothelium chambers on-chip with and without mechanical strain. (Error bars denote standard deviation of the mean, $n = 4$; n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.001$; $^{****}P < 0.0001$). *Figure reproduced with permission from Musah S, et al. Mature induced-pluripotent-stem-cell-derived human podocytes reconstitute kidney glomerular-capillary-wall function on a chip. Nat Biomed Eng 2017;1:1–12.*

mechanical stress and strain, compared to static cultures. Additionally, podocytes and endothelial cells both produce collagen under no-strain conditions, but under physiologically relevant strain, podocytes become the primary source of collagen IV production while endothelial cell collagen production decreases. This shift in collagen production is evidenced by immunofluorescence microscopic images in Fig. 24.6D and the histogram in Fig. 24.6E. Structural changes can also be observed under strain: podocyte foot processes extend through the engineered GBM (Fig. 24.5F), making contact with the laminin coated interface of the endothelium, as they do in vivo. These structural and functional observations reveal the sensitivity of podocytes to sheer stress and the importance of cyclic strain as a cue for podocyte development, in agreement with recent literature [122,123].

In light of the discovery that mechanical forces facilitate glomerular development, there have been few reports evaluating sheer stress within microfluidic devices that recapitulate the glomerulus. Of those available, FFSS within the device is calculated on the order of 0.001 [118] and 0.117 dyne/cm^2 [4], resulting from fluid flow rates through the chambers. Cyclic strain has only been reported by our lab and in one other study that utilized a mechanodynamic protocol similar to ours (10% cyclic strain, 1.5 dyne/cm^2 shear stress) used on mouse primary podocytes [126]. Additionally, existing literature suggests that, in excess, the same mechanical forces that drive nephrogenesis cause

podocyte injury and lead to CKD. A review by Kriz and Lemly in 2015 evaluated the potential role of mechanical forces on podocyte detachment and CKD progression, highlighting the lack of available literature on the topic and the need for more research [127]. Given our novel design of an in vitro glomerulus model, we now possess suitable tools to investigate the dynamic effects of mechanical forces on podocytes during development and degeneration in the future.

Testing of filtration function in an engineered model of the glomerulus

Kidney function is primarily evaluated by the efficiency of glomerular filtration processes, which include selective removal of toxins or small molecules (e.g., inulin) and retention of large molecules (e.g., albumin) in the blood. Measurement of two important molecules are indicative of kidney health: Inulin is a small exogenous molecule that is freely filtered by the kidney and therefore used frequently in the clinic to test kidney function; And leakage of albumin into the urine (also called albuminuria) is a clinical indicator of kidney injury or disease. In an in vitro system, selective molecular filtration and its efficiency are determined by measurements similar to those assessed in vivo. Simultaneous clearance measurements of total albumin and inulin offer information about the permeability and chemical selectivity of the filtration barrier. An inulin-and-albumin filtration assay is performed by perfusing fluorescently conjugated inulin- (10 μg/mL) and albumin- (100 μg/mL) containing media through the microvascular channel over 6 h while applying cyclic strain. The fluorescence intensity of the outflow from each channel was measured, and the amount filtered was calculated.

In vivo, it is estimated that at maximum efficiency, 6% of inulin is cleared from the blood stream into the urinary space, and virtually no albumin leakage occurs. In our glomerulus on a chip model, 99% of albumin was retained in the capillary compartment (even after 6 h of continuous perfusion), and 5% of inulin was filtered into the urinary channel (Fig. 24.6B). Importantly, this selective filtration of inulin and albumin was similar to in vivo estimates, which were not observed in the absence of adherent cells or when endothelium cells alone were cultured onto the microfluidic chip. Furthermore, differential clearance of molecules was also not achieved when other cell types, such as fibroblasts or renal proximal tubule epithelial cells (RPTECs), were cocultured with endothelium cells in the place of podocytes, as shown in Fig. 24.6C. These findings suggest that the interface of cocultured podocytes and endothelial cells mimics the normal filtration barrier of a functional glomerulus.

In vitro model of drug-induced glomerular injury and proteinuria

Human glomerular disease often manifests at the interface of the endothelial-podocyte tissue interface evidenced by the dysfunction or loss of these cells. It remains challenging to model human kidney disease in animal models or by using established immortalized podocytes, as these systems do not accurately depict the human physiological responses. Thus, the capability of an in vitro recapitulation of the human glomerulus to model injury and disease with integrity makes organ-on-a-chip tissue culture and analysis platforms a groundbreaking advancement for biomedical engineering and its applications in the field of nephrology. Glomerular disease states can be induced mechanically, pharmacologically, and genetically. Phenotypic changes following induction of a disease state are typically evaluated by alterations in gene expression, inefficient or nonselective filtration processes, and structural analysis of cellular morphology (i.e., effacement of podocyte foot processes), among other methods. In this section, we will discuss how our group and others have modeled glomerular disease and the tools employed to reflect phenotypic evidence of dysfunction.

In our in vitro model, we pharmacologically induced albuminuria by administering the cancer drug Adriamycin (ADR) [10]. We evaluated ADR-induced albuminuria by preforming the fluorescence-based filtration efficiency assay (described in the previous section) in a dose-dependent fashion. Dose-dependent responses were assessed for both nonselective leakage of albumin and inulin into the urinary channel and albumin reuptake into podocytes, as shown in Fig. 24.7D and E. By visualizing podocytes both with phase contrast and fluorescence microscopy images (Fig. 24.7A and B), we also found dose-dependent delamination of ADR-treated podocytes from the flexible ECM-coated PDMS membrane, in agreement with a dose-dependent loss of cell viability (measured with a CCK-8 viability assay). Interestingly, significant loss of podocytes was found both at clinically relevant doses and at lower doses of ADR, highlighting the sensitivity of podocytes to the drug (Fig. 24.7C). Intriguingly, we observed loss or retraction of podocyte foot processes in the podocytes cultured in the microfluidic devices that were treated with ADR (Fig. 24.7F). Together, these results show that podocytes seeded in the microfluidic device mimic the in vivo response to injury-inducing pharmaceutical stimuli.

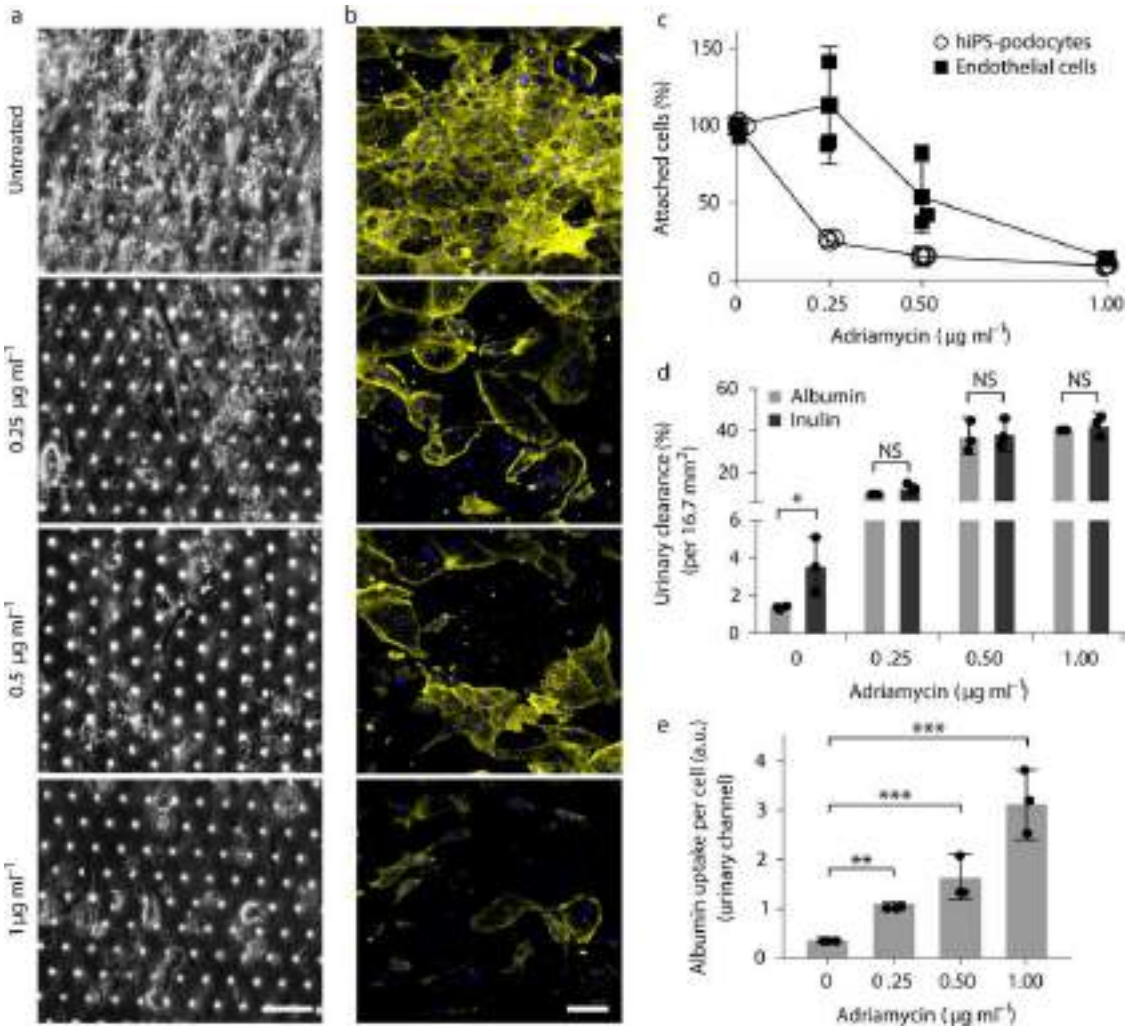

FIG. 24.7 Pharmacologically induced kidney glomerular injury in a microfluidic glomerulus-on-a-chip model. (A) Phase contrast and (B) Fluorescence microscopy images of human iPS cell-derived podocytes on-chip after infusion for 5 days with various concentrations of ADR through the vascular channel. Cells were stained with phalloidin (*yellow*) and counterstained with DAPI (*blue*). Scale bars, 100 μm. (C) Cell viability after 5 days of ADR infusion determined by quantification of podocytes and endothelial cells attached to their respective channels in the microfluidic device. (D) Urinary clearance assessment of albumin and inulin following 5 days of ADR infusion at various concentrations. (E) Quantification of albumin uptake by podocytes after 5 days of ADR-induced injury at various concentrations. (Error bars = standard deviation, $n = 3$; n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.001$; $^{****}P < 0.0001$). (F) SEM images of elongated foot processes in nontreated podocytes and podocytes treated with 0.5 μg/mL ADR. Scale bar, 10 μm. *Figure reproduced with permission from Musah S, et al. Mature induced-pluripotent-stem-cell-derived human podocytes reconstitute kidney glomerular-capillary-wall function on a chip. Nat Biomed Eng 2017;1:1–12.*

Other studies that employed glomerulus-on-a-chip technologies also modeled kidney diseases including diabetic nephropathy, glomerular hypertension, and membranous nephropathy (MN). Both Da Sacco and colleagues and Qin and colleagues induced diabetic nephropathy by administering glucose to the vascular chambers within their respective in vitro models and evaluated pathological changes to primary and immortalized podocytes, endothelial cells, and the interface between the two [4,117]. Da Sacco and colleagues further highlighted the versatility of their device for disease modeling by inducing focal segmental glomerulosclerosis (FSGS) via administration of puromycin aminonucleoside and MN via exposure to serum from MN patients and by deriving podocytes from patients with Alport Syndrome [4]. Lin and colleagues also induced glomerular hypertension by increasing the flow rate two- or threefold within their microfluidic device, thus increasing the FFSS experienced by adhered cell types [118]. The effects of each disease state were evaluated primarily by membrane permeability to albumin, inulin, and IgG, as described in the previous section, with increased leakage of each marker indicative of tissue damage. Cell viability was also tested, as well as structural evidence in the cytoskeleton and intercellular junctions of defacement of podocytes and endothelial cells, respectively. Evaluation of relevant biomarkers reveals vital information about the health of the tissue system. For example, during disease states, overexpression of C3d (protein derivative of complement component 3,

activation of complement pathway in immune system) [4], SNAIL (zinc-finger transcription factor SNAI1, expressed during kidney injury) [4], and GLUT1 (glucose transporter 1, transports extracellular glucose into the cell) [117] are observed, while ZO-1 (Zonula occludens-1, tight junction protein) [117] and WGA (Wheat germ agglutinin, an endothelial glycocalyx protein that serves as a protective lining in blood vessels) are underexpressed [4]. Furthermore, an increase in reactive oxygen species (indicative of oxidative stress) is thought to be an early indicator of disease state and was measured following glucose-induced diabetic nephropathy. Together, studies completed by us and others showcase the power of an in vitro recapitulation of the human glomerulus and the application of such technologies for disease modeling.

Conclusions and outlook

In this chapter, we provided a detailed overview of an established protocol for the derivation of kidney podocytes from human iPS cells. We outlined the important aspects of mammalian glomerulogenesis and development of the glomerular filtration barrier and how understanding of these processes could inform strategies for in vitro stem cell differentiation into specialized kidney cell types. We then discussed key considerations when trying to engineer a model of the kidney glomerulus – some of these factors include matrix mechanics and the roles of soluble signaling factors for guiding cell lineage commitment. Finally, we described our approach toward the establishment of a stem cell-derived glomerulus-on-a-chip platform that recapitulates the tissue-tissue interface, selective molecular filtration function, and drug-induced kidney injury and proteinuria in vitro. We envision that this work could improve current understanding of kidney development and disease mechanisms and facilitate the discovery of novel therapeutics and disease biomarkers.

This work also offers opportunities for regenerative medicine in the field of nephrology by enabling the production of functional cells and tissues that could potentially replenish damaged ones in vivo in the future. Advancements in stem cell therapy have been made possible by the implementation of robust protocols for the directed differentiation of human iPS cells into several disease-relevant cell types [80]. So far, efforts toward stem cell therapy in humans have been explored for treatment of a variety of vascular, cardiac, immunological, and renal diseases, among others, [9] while the field of regenerative engineering is still emerging.

One of the potential applications of our stem cell-derived technology is the possibility of developing isogenic cellular disease models that can help unravel disease pathogenesis, validate candidate therapeutics, and examine the nephrotoxicity of pharmacological and environmental agents. For example, almost every glomerulopathy, including nephrotic syndrome, minimal-change disease, FSGS, Immune Complex–Mediated Crescentic Glomerulonephritis, IgA, MN, HIV-associated nephropathy, hypertension, and APOL1 nephropathy disrupt the normal functioning of the glomerular filtration barrier. However, due to physiological differences and divergent pathways in organ development and function between humans and model organisms, it has not been possible to fully understand human kidney disease mechanisms. Integration of genome engineering technologies with our podocyte differentiation strategy can provide multiple disease modeling platforms with which to study disease-relevant mutations. These cellular disease models can be used to investigate pathophysiology throughout the course of GBM development (described in Section "Glomerular basement membrane") and can be a valuable resource for potential cell replacement therapies in patients suffering from CKD.

There have been several attempts to develop a molecular understanding of the glomerular filtration barrier. However, high abundance protein deposits, including albumin, immunoglobulins, complement proteins, and other blood proteins, and cell types, such as proximal and distal tubule cells and collecting duct cells, interfere with the proteome analysis of podocytes and the filtration barrier. We believe our glomerulus-on-a-chip device, with only the major cell types that compose the GBM, is well-suited to characterize disease-specific posttranslational modifications. Our device can also be used to isolate podocytes specifically from the top channel with very high cellular fidelity and visualize genes and proteins in a spatial context to help map important regulatory pathways to identify, quantify, and obtain a systems-level understanding of the glomerular filtration barrier.

Kidney diseases have attracted growing commercial interest due to the lack of high-quality pharmaceutics, but the development of new drugs for genetic disorders has been impeded due to scarcity of patient populations. Moreover, the populations affected by kidney diseases vary greatly in terms of genetic predispositions, diet, and socio-economic status, which makes it difficult to identify personalized biomarkers linked to the disease or drug response. In preclinical drug discovery trials, pharmaceutical testing in animals does not accurately reflect human drug reactions due to species-specific biochemical and pharmacokinetic differences. Additionally, analyses of large clinical trials for population-specific drug discovery is costly. We believe our glomerulus-on-a-chip system could provide an intermediate step

to screen pharmaceutical leads prior to human trials. To this end, the microfluidic device can be utilized to uncover molecular underpinnings of variability in drug responses between individual patients or specific populations.

More recently, researchers have extended organ-on-a-chip technology to create interconnected organ mimetic systems (or 'body-on-chip). For example, Ingber and colleagues developed a multiple organ-on-a-chip device to simulate systemic function of the human body to predict pharmacokinetics [128,129]. Such body-on-a-chip microfluidic platforms mimicking physiologically relevant heterotypic (between different cell types) and homotypic (between same cell types) interactions can be used to assess organ development and disease progression for a particular genetic predisposition. These integrated systems require fluid handling capabilities in a controlled manner to ensure adequate supply of nutrients and oxygen and enable systematic removal of cellular waste metabolites and toxins and diffusion of protein factors. In the future, we predict these fluidically linked multiorgan systems could be designed and built such that they are equipped with built-in molecular sensors and automated fluid handling capabilities to enhance timely characterizations of biochemical processes.

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CHAPTER

25

Microfluidic modeling of the glomerulus and tubular appartus

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Introduction

The discovery and development of new drugs are a significant challenge. Reports from the Centers for Medical Research (CMR) show that less than 1 out of every 10 drugs entering clinical trials makes it to market, with most compounds failing due to a lack of efficacy [1]. Although the average cost to bring a new drug to market has recently decreased to less than $2 billion [2], and the average time to market has decreased from an average of 14.5 years in 2010 to 10.2 years in 2019 [3], bringing a new drug to market remains a long, complex, and costly process. The expense of developing new drugs and the large number of new compounds available to be tested makes a rapid, inexpensive, and accurate method for assessing efficacy and toxicity in humans essential to the pharmaceutical industry.

The kidneys are responsible for filtration, osmoregulation, and reuptake and receive ~ 25% of cardiac output, meaning that they play a key role in drug adsorption, distribution, metabolism, and elimination (ADME) [4]. Pharmaceuticals as well as exposure to some environmental or industrial chemicals or toxins, can result in nephrotoxicity that leads to acute kidney injury (AKI) and an increase in patient morbidity and mortality [5, 6]. Up to 20% of AKI is thought to be associated with drug-induced nephrotoxicity [5, 7]. Kidney toxicity is also one of the most widely reported adverse effects during drug development, is often not detected until late in the process, and is second only to liver toxicity in causing clinical trials to fail [8]. This highlights the importance of understanding interactions between the kidneys and new drug candidates.

Kidney models currently used in preclinical studies are not able to successfully replicate the structure and function of the kidney, and therefore poorly predict drugs that induce nephrotoxicity. In vitro cell culture systems are a widely used experimental tool during the preclinical phase. Human kidney cells are generally cultured in static, two-dimensional (2D) plastic plates, which are fast, inexpensive, easy to manipulate, and allow for high-throughput assays. Cell cultures are limited in their ability to predict drug toxicity and efficacy, however, because they lack the physiological stimuli and tissue architecture found in vivo [9]. 2D, single cell-type cell cultures cannot recreate the human body's microenvironment, including cell-cell and cell-matrix interactions, nutrient and oxygen supply, waste removal, and interaction with other organs and tissues through soluble proteins or metabolites [10].

Animal studies are the primary method used to determine new compound toxicological and pharmacological profiles, but animal experiments are long, expensive, lack the ability to study single factor changes, and drug interactions with animal tissues can differ significantly from human tissue interactions. There are significant species differences in the expression, substrate specificity, tissue distribution, and relative abundance of drug transporters important to drug-induced nephrotoxicity (e.g., [11, 12]). Approximately, 2% of drugs that are safe for use in animals prove safe for use in humans [13, 14]. This is an ethical dilemma, for both the use of animals and for the human patients involved in clinical trials. Organ on a chip or microphysiological systems (MPS) can overcome some of the in vitro cell-based and in vivo animal model shortcomings.

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The combination of microfabrication, microfluidics, and tissue engineering technology have enabled the creation of MPS. The simplest systems involve the growth of one or more cell types in a single, perfused microfluidic chamber to exhibit the functions of one tissue type. More complex devices incorporate multiple cell types and aim to replicate human physiology with respect to the size relationships of organs, blood distribution, and blood flow. When operated with human-derived cells, these systems are capable of replicating human drug metabolism, therapeutic action, toxicity, and functional tissue responses. The goal of MPS is to provide more physiologically realistic microenvironments for and interactions between human cells, allowing them to better recreate tissue and organ level functions in an easily built and manipulated apparatus. MPS have the potential to direct drug development toward the most promising candidates or allow other compounds to fail fast, i.e., prevent unsafe compounds from entering human clinical trials (Fig. 25.1). The goal of this chapter is to provide an overview of the current state of kidney microfluidic models, design considerations in developing kidney devices and current applications of the technology.

Current microphysiological models of the kidney

Introduction to MPS

One of the first systems of interconnected tissue compartments was developed in 1995 using tools and concepts from biochemical reactors. The system was constructed with large glass dilution bottles and commercially available tubing [15, 16]. The reactor was filled with rat liver and lung cells, and was used to show that naphthalene was metabolized by the liver cells and the resulting metabolite was toxic to lung tissue. Current MPS devices have evolved to use microfabrication, micromachining, or 3D printing (see section "Design considerations") for device fabrication. The much smaller scale provides controlled, laminar fluid flow, a liquid-to-cell ratio that is closer to that found in the body, and a low overall volume that requires less valuable cells and/or valuable reagent. The dynamic environment can improve cell differentiation and function, microfabricated tissue scaffolds can provide a more in vivo-like architecture, and coculture of multiple different cell types can better recreate the complexity of human tissues.

The motivation behind the initial body on a chip devices was to create a physical representation of physiologically based pharmacokinetic (PBPK) models. PBPK models are mathematical models of human metabolism that take into account physiologic parameters such as organ sizes, blood flow rates through organs, and enzyme activity to predict the ADME of new drugs. PBPK drawings can inform the layout of body on a chip systems, body-on-a-chip devices replace equations with actual enzymatic reactions taking place within the organ compartments and fluid dynamic equations with fluidic flow within microfluidic channels. This relationship allows for the ability to build a very accurate PBPK model of the body-on-a-chip device [17], and Abaci and Shuler have provided a useful template for the design of PBPK-based MPS [18]. Mathematical models, together with single organ on a chip devices, can also be used to obtain tissue-specific parameters that were previously unknown [18, 19]. With respect to renal system, MPS have been developed to model both normal and diseased renal physiology. These models have been used to demonstrate drug-induced nephrotoxicity from compounds known to induce AKI, but may at some point be predictive. Results with these models are summarized in Tables 25.1 and 25.2.

Glomerulus MPS

The glomerulus, a network of capillaries in a cup-shaped structure (Bowmańs capsule), filters out solutes from the blood based on charge and size to create glomerular filtrate. Glomerular filtration consists of passive movement of plasma from the glomerulus capillaries to the Bowmańs capsule, known as ultrafiltration. Within the endothelium, fenestrations are freely permeable to water and small molecules/solutes (Na^+, urea, and glucose), but not to blood, large proteins, white blood cells, and platelets [36]. While small molecules are easily eliminated by the kidneys, large molecules (> 67 kDa) remain in the bloodstream. Failure in these fundamental filtration processes propagates glomerular diseases, such as glomerulonephritis and glomerulosclerosis. Developing an in vitro microphysiological system that captures the specificity of glomerular function proves essential in defining underlying factors involved in glomerular pathophysiology.

Zhou et al. developed the animal, diseased glomerulus model of hypertensive nephropathy. Immortalized mouse glomerular endothelial cells and murine podocyte precursor cells were cultured and arranged in layers lining two channels. From the outcome, it successfully replicated cytoskeletal rearrangements, cellular damage, and glomerular leakage by fluid flow. The work demonstrated the primary backbone for disease modeling within a human glomerulus [20].

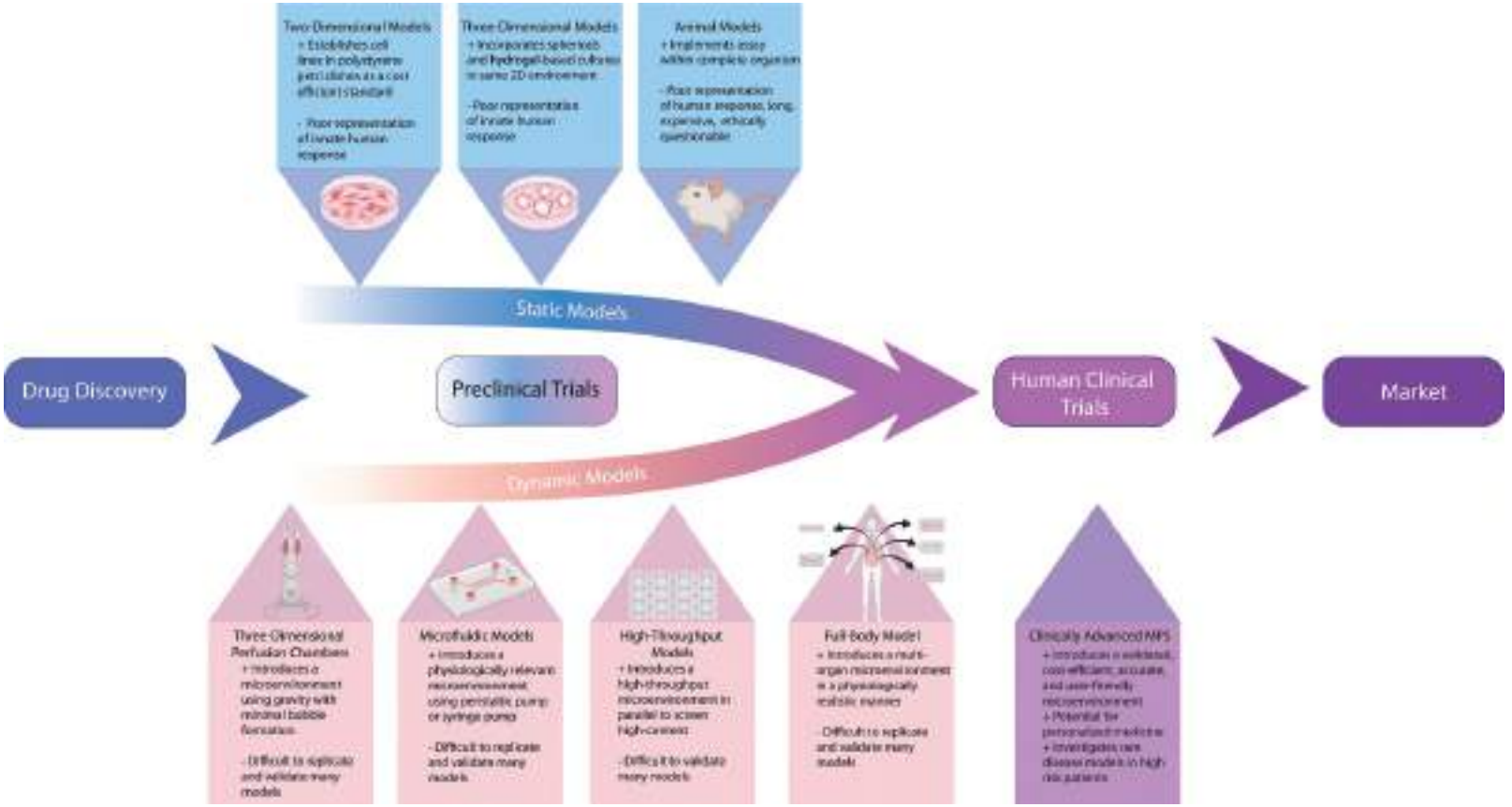

FIG. 25.1 Current and future methods for assessing drug safety.

TABLE 25.1 Microphysiological system cellular models and key findings.

Kidney structure	Cell type	Fluid flow duration	Model type	Key findings	References
Glomerulus	Mouse glomerular endothelial cells Murine podocyte precursor cells	24 h	Disease (hypertensive nephropathy)	FSS led to cytoskeletal rearrangement; mechanical forces damage cells and cellular junctions	[20]
	Rat glomerular endothelial cells and podocytes	2 weeks	Disease (diabetic nephropathy (DN))	Recapitulated the critical pathological responses induced by high glucose conditions that are characteristic of DN	[21]
	Human-induced pluripotent stem cells-derived podocyte-like cells	8 days	Healthy diseased (adriamycin-induced podocyte injury and albuminuria)	Cells secreted glomerular basement membrane collagen and recapitulated the natural tissue-tissue interface of the glomerulus, differential clearance of albumin and inulin	[22]
	Human primary podocytes (hpPOD) Human immortalized podocytes (hiPOD) Amniotic fluid-derived podocytes (hAKPC-P)	28 days	Healthy diseased (membranous nephropathy and glucose-induced damage)	OrganoPlate platform with a coculture of human podocytes and human glomerular endothelium cells (hGEC) without the reliance of artificial membranes	[23]
Proximal convoluted tubule	Human primary renal proximal tubule epithelial cells (PTECs)	3 days	Healthy disease (nephrotoxicity—cisplatin)	In vitro barrier proximal tubule microdevice; comparison of fluid shear stress (FSS) and static flow; cytoskeletal rearrangement in FSS; mechanical forces play a role in the damage of cells and cellular junctions	[24]
	Human primary renal proximal tubule epithelial cells (PTECs)	7 days	Healthy	Proximal tubular structure seeded with monolayer of cells under FSS showed functional capabilities	[25]
	Opossum kidney (OK) cells Madin-Darby canine kidney epithelial (MDCK) cells Porcine kidney epithelial (LLC-PK1) cells	3 h	Healthy disease (proteinuria)	Modulation of GFR and the fluid shear stress (FSS) cause acute changes in ion transport, apical endocytosis, and primary cilia	[26]
	Adult renal stem/progenitor cells (ARPCs)	6 h	Healthy	Proximal tubule stem cells within microfluidic system; expressed similar FSS impacts	[27]
	Human conditionally immortalized proximal tubule epithelial cells (ciPTEC)	30 min	Healthy	Present reabsorption and secretory functions in clearance of albumin-bound uremic toxins in hollow fibers of proximal tubule cells	[28]
	Human primary renal proximal tubule epithelial cells (PTECs)	4 weeks	Healthy	Long-term FSS microdevice with cells to form a tubular structure; more assays (GGT activity, ammoniagenesis, vitamin D metabolism, secretory transport) validated physiological relevance	[29]
	Human primary renal proximal tubule epithelial cells (PTECs)	2 months	Healthy diseased (nephrotoxicity—cyclosporin A)	Bioprinted tubular structure and long-term study; exhibited a concentration-dependency after exposed to cyclosporin A	[30]
	Human primary kidney cells (HIM-31) Human renal proximal tubule epithelial cells (RPTECs)	21 days	Healthy diseased (nephrotoxicity—polymyxin B)	Long-term case study of proximal tubule microphysiological system	[31]
	Human renal proximal tubule epithelial cells (RPTECs)	24 days	Diseased (nephrotoxicity)	Tested renal absorption in five chemical compounds: creatinine (negative control), perfluorooctanoic acid (positive control), cisplatin, gentamicin, and cadmium	[32]
Glomerulus and proximal tubule	Human kidney proximal tubule cells (HK-2) Human umbilical vein endothelial cells (HUVECs)	2 days	Healthy	Reusable glomerulus and proximal tubule microdevice; introduction of new assays (ALP, NGAL, and fibronectin excretion)	[33]
	Conditionally immortalized human podocytes (CIHP-1) Human kidney proximal tubule cells (HK-2) Human umbilical vein endothelial cells (HUVECs)	7 days	Healthy	Reusable glomerulus and proximal tubule microdevice; introduction of computational model and biological and mechanical representation of filtration	[34]
Collecting duct	Primary rat inner medullary collecting duct (IMCD) cells	5 h	Healthy	Collecting duct microdevice; detection of cell polarization and rearrangement of cytoskeleton and cell junctions	[35]

TABLE 25.2 Microphysiological system features and assays.

Kidney structure	Material	Dimensions	Fabrication method	Assays of physiological relevance	References
Glomerulus	PDMS (bi-layer) Polycarbonate (membrane barrier)	Main channel: 104 μm × 1000 μm × 100 μm Width of outflow channel: 300 μm Width of inflow channel: 1000 μm	Photolithography Soft lithography	Immunofluorescence staining CD-31, F-actin, vWF in GEnCs, synaptopodin, F-actin, nephrin, podocin Permeability FITC-inulin, FITC-BSA, FITC-IgG Protein filtration β2-microglobulin, microalbumin, and IgG	[20]
	PDMS (bi-layer)	Five microchannels (1) Capillary lumen channel (2) Matrigel channel (3) Capsule channel Height of vascular channel = 3 × height of matrigel channel	Photolithography Soft lithography	Permeability TRITC, FITC-IgG SEM Protein filtration Albumin Immunofluorescence staining CD-31, GLUT-1, ZO-1, synaptopodin, vimentin Cell viability Calcein AM and Eth-D Cell migration Glucose recovery test	[21]
	PDMS (bi-layer) PDMS (membrane barrier)	Top channel (urinary): 1 × 1 mm Bottom channel (microvascular): 1 × 0.2 mm Membrane: 50 μm thick, 7 μm diameter pores, 40 μm spacing	Stereolithography Soft lithography DRIE	Protein filtration FITC-Inulin, Alexa Fluor 555-albumin Cell viability LDH release SEM Flow cytometry Nephrin, WT1, Oct4 Immunostaining β1 integrin, Oct4, Tra-1-60, Goosecoid, Hand1, Pax2, nephrin, podocin, OSR1, VE-cadherin, PKC	[22]
	Polystyrene, glass, proprietary polymers	OrganoPlate culture: 40 networks in 96-well plate Three-lane chip (1) Phaseguide: 100 μm × 55 μm × 220 μm (2) Gel channels: 9 mm × 350 μm × 220 μm (3) Perfusion channels: 13 mm × 300 μm × 220 μm	Commercial purchase (Mimetas) Etching	Protein filtration FITC-inulin, FITC-albumin SEM Flow cytometry Nephrin, WT1, Oct4 Toxicity/recovery Test PAN, glucose Immunostaining CD24, OB-cadherin, Podocalyxin, CD31, VEGFR2 (see Table 25.1)	[23]

Continued

TABLE 25.2 Microphysiological system features and assays—cont'd

Kidney structure	Material	Dimensions	Fabrication method	Assays of physiological relevance	References
Proximal convoluted tubule	PDMS (bi-layer) Polyester (membrane barrier)	Device: 1.1 cm × 1.1 mm × 3 mm Channel: 1 cm × 1 mm × 100 mm Membrane: 0.4 mm pores, 10 mm thick	Photolithography Soft lithography	Protein filtration FITC-albumin Cell viability Pgp transporter activity, LDH release Western Blot ZO-1, AQP1, Na/K-ATPase, GAPDH, SGLT2, and α-tubulin Toxicity/recovery test Cisplatin Immunostaining ZO-1, AQP1, and Na/K ATPase	[24]
	PDMS and glass (body) PES and PVP (tubular structure)	Tubular structure: OD (780 μm), ID (490 μm), and fiber thickness (145 μm) ID pore size: 0.5 μm OD pore size: < 10 nm Dope composition: 18% PES/10% PVP/72% NMP	Commercial purchase Extrusion	Protein filtration FITC-inulin, albumin, albumin + ouabain Immunostaining γ-GTP, AQP1, CD13, Na/K-ATPase, TRITC-IgG, phalloidin, and MitoTracker, Hoescht SEM Toxicity/recovery test Urea, creatinine, glucose	[25]
	Glass slide	Six-well chambers Channel: 17 × 3.8 × 0.4 mm	Commercial purchase (Ibidi)	Protein filtration Alexa Fluor 647-BSA, lysine-fixable rhodamine-dextran Toxicity/recovery test Calcium concentration, ammonium sulfate Immunostaining Antiacetylated tubulin	[26]
	PDMS (bi-layer) Polycarbonate PC (membrane)	Channel: 500 × 120 mm Membrane pore size: 0.1 mm Membrane thickness: 40 mm	Photolithography Soft lithography	Protein filtration Rhodamine, dextran Immunostaining phalloidin-TRITC, DAPI, ZO-1, AQP2, Na/K + ATPase pump	[27]
	Polycarbonate Polyester (PET) Polytetrafluoroethylene (PTFE)	Transwell inserts	Commercial purchase	Protein filtration FITC-inulin Cell viability MTT Toxicity/recovery test Indoxyl sulfate, kynurenic acid Immunostaining OAT1, BCRP, and MRP4	[28]

	Polyurethane (cast-molding) PDMS (bi-layer) Membrane (silicone septa and microfibers)	Tubular structure: 6 mm long × 120 mm thick	Commercial purchase (Nortis) Micromilling Cast-molding	Protein filtration Alexa Fluor 647-BSA, lysine-fixable rhodamine-dextran Cell viability ATP assay, GGT activity Functional assays Ammoniagenesis, glucose reabsorption (fluorescent glucose analog 2-NBDG), and vitamin D metabolism Toxicity/recovery test antimycin A, indoxyl sulfate	[29]
	Gelatin-fibrinogen matrix	Max table: 725 × 650 × 125 mm Glass slide: 50 × 75 mm Nozzle: 410 μm	Commercial purchase 3D Bioprinter (Aerotech Inc.)	Protein filtration Alexa Fluor 647-BSA, lysine-fixable rhodamine-dextran Toxicity/recovery test Antimycin A, indoxyl sulfate Immunostaining AQP1, Na/K ATPase, cilia, nuclei	[30]
	PDMS (bi-layer) Membrane (microfiber filament insert)	Tubular structure: 6 mm long × 120 mm thick	Commercial purchase (Nortis)	Protein filtration Alexa Fluor 647-BSA, lysine-fixable rhodamine-dextran Cell viability ATP assay, GGT activity Functional assays Ammoniagenesis, glucose reabsorption (fluorescent glucose analog 2-NBDG), and vitamin D metabolism	[31]
	PDMS (bi-layer) Membrane (microfiber filament insert)	Tubular structure: 6 mm long × 120 mm thick	Commercial purchase (Nortis)	Toxicity/recovery test Creatinine, PFOA, cadmium, gentamicin, and cisplatin Protein binding assays RED Extrapolation model	[32]
Glomerulus and proximal tubule	Polycarbonate (bi-layer) Polycarbonate (tubular membrane) Silicone (gasket) PES (glomerular membrane)	Glomerular channel: 14 mm diameter Proximal tubule channel: 15 mm × 30 mm × 50 μm	CNC machining	Protein filtration BSA, ovalbumin-Alexa Fluor 488 Cell viability GGT activity, ALP phosphatase Immunostaining Caveolin-1, occludin, ZO-1, clathrin, and β-catenin Functional assays NGAL/fibronectin excretion	[33]
	Polycarbonate (bi-layer) Polycarbonate (tubular membrane) Silicone (gasket) PES (glomerular membrane)	Glomerular channel: 14 mm diameter Proximal tubule channel: 15 mm × 30 mm × 50 μm	CNC machining	Functional assays Preurine formation Glucose secretion Protein filtration FITC-human serum albumin Fluorescent staining	[34]
Collecting duct	PDMS (bi-layer) Polyester (membrane barrier)	Device: 1.1 cm × 1.1 mm × 3 mm Channel: 1 cm × 1 mm × 100 mm Membrane: 0.4 mm pores, 10 mm thick	Photolithography Soft lithography		[35]

Wang et al. designed the animal, diseased glomerulus model of diabetic nephropathy through culturing rat glomerular microtissues. The 2D architecture of the microdevice consisted of five interconnected channels to mimic the Bowmaṅs capsule, glomerular basement membrane, and podocyte layer interface. The disruption of endothelial cell and podocyte interactions through changes of barrier integrity and permeability in the presence of high glucose were observed. This diseased model showed the potential for the establishment of more diseased kidney models, which would be able to look at disruption along different parts of the nephron [21].

Musah et al. established the first human, healthy glomerulus model, using terminally differentiated podocytes from human-induced pluripotent stem cells. Their system mimicked the tissue-tissue interface and molecular filtration properties of the glomerular capillary wall. In the presence of doxorubicin, podocyte death and proteinuria were observed in the microfluidic system [22].

Petrosyan et al. featured the OrganoPlate platform with a coculture of human podocytes and human glomerular endothelium cells (hGEC) without the reliance of artificial membranes. Three types of human-derived podocytes were compared: (1) human originated primary podocytes obtained from discarded, healthy kidneys (hpPOD), (2) immortalized podocytes (hiPOD), and (3) amniotic fluid-derived podocytes (hAKPC-P). In addition to establishing a healthy model, the system was validated from glomerular diseased conditions, including membranous nephropathy (MN) and glucose-induced damage. The work suggests a functional glomerular filtration barrier (GFB) for long-term evaluation (1 month) [23].

While glomerular microfluidic systems are limited based on a myriad of available human-derived cell models, reproducibility, and directionality of the flow, these functional models are mimics of both healthy and diseased states, critical steps into building human microphysiological systems for drug screening and increasing our understanding of the cellular and molecular mechanisms responsible for glomerular injury and podocyte loss.

Proximal convoluted tubule MPS

Following Bowmaṅs capsule, glomerular filtrate exits the glomerulus and proceeds to the renal cortex, a segment of highly coiled tubules. Overall, the tubular system undertakes influx, efflux, and intracellular metabolism through a series of solute carrier transporters, regulating both xenobiotics and endogenous substrates in the blood. Of the tubules, the proximal convoluted tubule is characterized by the apical (reabsorption) and basolateral (absorption) membranes that employ active and passive transport to reabsorb sugar, sodium chloride, and water from the glomerular filtrate. Extensive studies have been conducted within this region due to its role in controlling glomerular filtration rate, drug transport, and metabolism. The ability to control reabsorption and secretion of molecules is a critical aspect of accurately reproducing the proximal tubule function in vitro. Recapitulating proximal tubular function is particularly important for studies on drug-induced nephrotoxicity, as drug clearance, reabsorption, intracellular concentration, and local interstitial accumulation all primarily occur at this site in the kidney [37,38].

Jang et al. generated an in vitro barrier model of a human proximal tubule through a porous membrane seeded with human primary renal proximal tubule epithelial cells (PTECs) on the apical (luminal) layer. Under fluidic conditions of 0.2 dyne-s/cm^2, proximal tubule cells gained polarity, columnar shape, primary cilia, and functional transporters, central for mechano-sensing and regulation of tubular morphology in vivo, while the same cells under static conditions failed to experience this. Series of experimental assays assessing the secondary active transport function of the cells, the uptake of albumin, active glucose transport, and alkaline phosphatase (ALP) in presence of cisplatin were measured and established the gold standard in characterizing kidney-based microdevices for toxicity testing [24].

Ng et al. introduced a hollow fiber bioreactor, seeded with PTECs on the inner surface coated with fibrin. The system was the first to generate a tubular structure under fluidic shear stress with a monolayer of proximal tubule cells, possessing functional transport capabilities. Inulin recovery (urea, creatinine, and glucose) and protein (inulin, albumin, and ouabain) assays were implemented to validate its functionality and compared to static conditions [25].

Raghavan et al. cultured three animal-derived kidney cell lines onto parallel flow chambers and subjected to a flow rate of 1 dyne-s/cm^2. From the work, they identified how changes in GFR and the fluid shear stress (FSS) modulate acute changes in ion transport, apical endocytosis, and primary cilia [26].

Sciancalepore et al. differentiated human renal stem/progenitor cells in a microfluidic system. Under flow of 1 μL/min, the cells polarized and their permeability of urea and creatinine decreased, different to cells cultured under static conditions [27].

Jansen et al. incorporated ciPTECs into biofunctionalized hollow fibers, in which both reabsorption and secretory functions were denoted through the clearance of albumin-bound uremic toxins and albumin [28].

Weber et al. subjected human PTECs into a Nortis microphysiological system to form a tubular-like structure for 4 weeks. Similarly, FSS generated changes in cell polarization, cellular morphology, basolateral and apical transport,

and intracellular enzymatic function through a series of assays (GGT activity, ammoniageneis, and vitamin D metabolism) [29].

Homan et al. bioprinted a complex tubular architecture circumscribed by PTECs under flow of 0.1–0.5 dynes/cm^2 for 2 months. Results proved that cells responded morphologically (polarization of epithelium) and functionally (albumin reuptake) like that of in vivo conditions and exhibited a concentration dependency after exposed to cyclosporin A (drug toxicity). Bioprinting was validated as a viable option for FSS-induced kidney-based studies [30].

Sakolish et al. utilized a Nortis Bio microphysiological system with primary human kidney cells (HIM-31) and human renal proximal tubule epithelial cells (RPTECs) under FSS of 0.25 dynes/cm^2 for 21 days [31]. This work demonstrated a case study of technology transfer from Weber et al. [29] to an independent laboratory setting, which established extended FSS duration in assessing two different cell types and employed drug toxicity assays. Sakolish et al. furthered the prediction of tubular reabsorption of the same system through assessment of five additional chemical compounds: creatinine (negative control), perfluorooctanoic acid (positive control), cisplatin, gentamicin, and cadmium. The system provides a better mechanistic understanding of renal injury and its dynamic effects on transport and clearance [32].

Glomerulus and proximal tubule MPS

In most pharmacokinetic models, chemical components not only target individual structures, but also multiple platforms. Within the kidney, this is represented in series with both glomeruli and tubular structures. However, with multilevel devices, higher complexity and difficulties arise regarding the shear rates and glomerular filtration.

Sakolish and Mahler established the first glomerulus and proximal tubule microphysiological system with HUVECs and HK-2 cell types for each respective structure. Comparisons of static and FSS conditions in both single channel and multilayer channel were conducted to properly model the removal of compounds that have been reabsorbed by the renal tubule and to prevent the effects of a concentration gradient [33]. Zhang and Mahler described a tri-culture of glomerular podocytes, human umbilical vein endothelial cells, and proximal tubule epithelial cells within a microphysiological system [34]. A single-culture MPS (only proximal tubule cells) was compared with that of the tri-culture model. Based on the results, the incorporation of the glomerular filtrating cells from the tri-culture biologically filtered human serum albumin. Therefore, a tri-culture presents a more realistic cellular model of the glomerulus and proximal convoluted tubule.

Since this establishment of glomerular and tubular coculture, there has been limited investigation in combining nephron structures. Several contributing factors stem from environmental impacts, such as medium adaptation, cellular adaptation, fluid directionality, and apparatus hindrances. With each human renal cell type (21 types), individualized nutrients and growth factors may differ in the presence of coculturing cells. Therefore, additional testing and careful selection of cellular models determine the success of viable cell-cell interactions. In glomerular and tubular structures, inclusion of fluid unidirectionality (glomerulus) and bidirectionality (apical and basolateral) alongside the incorporation of physiological shear rates (0.7–1.2 dynes/cm^2) [39,40] proves challenging. Common apparatus hindrances include the lack thereof, leakage, contamination, and bubble formation. In silico computational testing may overcome and minimize the mechanical constraints through geometric and volumetric parametrization, but system usability remains extensive for troubleshooting and iterative, especially for long-term studies. While current diagnostics for microfluidic kidney platforms are impaired and constrained, technology and our understanding of kidney physiology and pathophysiology are constantly propagating, serving as a beacon in innovating effective therapeutic agents.

Loop of Henle/distal convoluted tubule MPS/collecting duct MPS

Following the highly coiled proximal convoluted tubule, the Loop of Henle further reabsorbs water and sodium chloride to minimize the amount of water lost by the organism and produces a highly concentrated urine. Afterwards, the distal convoluted tubule regulates potassium, sodium, calcium, and pH levels in the filtrate [41]. Finally, the collecting ducts output the urine into the bladder for storage and eventual excretion out of the body [40,42,43]. Few groups have attempted to develop a distal tubule/collecting duct-on-a-chip model. Jang and Suh [35] achieved the first animal-based collecting duct multilayered microdevice with primary rat inner medullary collecting duct (IMCD) cells at FSS of 1 dyne-s/cm^2. Similarly, FSS cells experienced polarization and cytoskeletal reorganization.

Design considerations

2D glomerulus and tubular models have been prolific in studying cisplatin, the gold standard for toxicity tests in the kidney [38,44]. Within these 2D models and 3D organoids in polystyrene plates, the static conditions do not

polarize cells and demonstrate full functionality in a human kidney [24]. A functional cellular model under shear stress is a minimum requirement for an effective kidney microphysiological system for toxicity testing. Construction of these renal MPS models have widely impacted the way scientific studies are conducted. The fabrication techniques, cellular models, and material selection influence the type of 3D kidney model, the function, and its limitations. With its applications in stem cell research [22], regenerative medicine [45], biomaterials [46], tissue engineering [47], and microfluidics, MPS have furthered the fundamental backbones of renal sciences.

Fabrication

As technology advances, fabrication techniques progress toward constructing more effective and highly complicated systems. Microfabrication consists of manufacturing 3D scaffolds in a geometrically constrained environment through various techniques (photolithography, micromilling, etc.) [48].

Lithography

Traditionally, photolithography entails light energy passing through a patterned mask where an optical image is produced. Initial microfabrication techniques are implemented on silicon wafers, key semiconductor substrate for electromechanical systems. Through depositing a photoresist to create a photosensitive film, the pattern on a substrate is created as a master mold for soft lithography [49]. Soft lithography involves the fabrication of structures using elastomeric stamps or molds. The end goal is to generate a polymeric device that can load cells or possess electrical components [50]. Following, polymers, such as polydimethylsiloxane (PDMS), are cast into the master mold, solidified in high temperatures, and adhered onto a glass slide or thermoplastic polymer [51–53]. PDMS constructed through lithography has been utilized in several kidney models [20, 21, 24].

Micromachining

Though photolithography is typically done in the cleanroom, there are alternative techniques to overcome the financial burdens of maintaining these expensive tools and the limited accessibility. These include micromilling, such as computer numeric control (CNC) machining, and 3D printing. CNC is a subtractive manufacturing tool that utilizes computer technology to extract and carve material away [54]. The terminology for the incorporation of CNC in microfluidics is "micromilling." Many previous works have identified the effectiveness of micromilling in microfluidics, such as Kit-On-A-Lid-Assays (KOALA) [55], different assays [48], and RNA, DNA, and protein isolation [56,57]. Using a CNC machine to create an extruded mold for elastomeric stamping is one technique for micromachining [58]. However, as the hydrophobic nature of PDMS can interfere with toxicity assays, thermoplastic polymers become more attractive for long-term drug testing [59]. CNC machining polycarbonate devices have already been demonstrated in the glomerulus and proximal tubule MPS [31,33]. From these works, these techniques are inexpensive and provide rapid prototyping for high throughput [31].

3D printing

A 3D printer manipulates a solidified material under computer control to create a 3D object through additive manufacturing. Currently, there is stereolithography (SL), multijet modeling (MJM), and fused deposition modeling (FDM). SL exploits photopolymerization, a technique that uses a high-intensity laser to solidify a photoresin [60]. While this system is highly expensive, it employs multiphotonic optics to increase resolution specifications [61–63]. Similarly, multijet modeling (MJM) manipulates ultraviolet rays to cure liquid photopolymer. Fused deposition modeling (FDM) or "thermoplastic extrusion" manipulates thermoplastic material [64]. All printers are costly and biocompatibility and biofunctionality studies remain unknown [65]. However, bioprinting has been keystone in introducing 3D printing in renal studies. Current methods are attempting to create a more robust and functional model of the proximal tubule with a more convoluted architecture and open lumen. The specific and controllable amounts of shear stress on the epithelial cells tend to enhance the sensitivity of the cells to nephrotoxic drugs by exaggerating their differentiation and using physiological shear stresses to provide a more accurate assessment. 3D bioprinting is one technique used to address some of these deficiencies [30,66,67]. In this study [30], an ECM solution was created with 10 mg/mL of fibrinogen, 7.5% (wt) of gelatin, 2.5 mM of calcium dichloride, and 0.2% (wt) of transglutaminase solution to create the base layer of the 3D proximal tubule model [30].

Cells

In kidney MPS, the type of cells affects the functionality and realistic cell-cell interactions. Initially, animal-derived immortalized cell lines were prevalent for renal-based studies, but these do not reflect the human responses as

demonstrated in preclinical trials. Therefore, human-derived immortalized cell lines, such as human-kidney 2 proximal tubular cells (HK-2) and conditionally immortalized human podocytes (CIHP-1) cell lines, were selected for testing based on its low-cost and ease of maintenance. However, cell phenotype, lack of functional differentiation, and poor expression of human-specific transporters hinder the impact of the work.

An alternative is utilizing primary cell lines, such as human proximal tubule epithelial cells (PTECs), which have grown in popularity that have demonstrated increased transporter expression and functionality. The limitations of primary cell lines include the heterogeneous cell populations, cost, time-consuming isolation, and limited cell numbers due to cell senescence in culture. Differentiating podocyte-like cells from stem cell lines or induced pluripotent stem cells have been utilized to study renal function, but more characterization is required to confirm functionality [22].

Materials

Biocompatibility of a MPS is necessary for preliminary comparisons in a healthy renal model. Silicon devices are biocompatible, but require additional coatings with extracellular matrices to maintain confluency in tissue chambers. While PDMS is conventional for cell-based studies as inexpensive and biocompatible tools, hydrophobic drugs or metabolites are known to adsorb onto the material, rendering it unreliable for toxicity testing [59,68]. One way to remedy this problem is to coat the devices with a layer of Parylene C, which can significantly reduce drug and metabolite absorption into PDMS [69]. Additional materials for fabrication consideration include glass [26], thermoplastics [47], other polymers (polyester [35], polyethersulfone [33], and silicone septa [29]), and electrospun fibers of supramolecular, ureido-pyrimidinone (UPy) modified polymers [70,71].

Applications

Clinical use

Integration of the kidney MPS into a multiorgan systems could enable the study of inflammatory response, systemic and secondary toxicity of drugs [72]. In preclinical drug screening studies, kidney MPS can be used to assess both toxicity and confirm effectiveness in treating the intended target pathways. New drug candidate advancement through clinical trials could be expedited by incorporating MPS [73]. MPS developed with a patient's own cells provide a method for testing the effects of new medications on rare diseases, high-risk populations, and/or diverse groups. Use of MPS in this manner could, for example, enable development of individualized therapeutic regimens, help uncover therapeutic targets, allow effective repurposing of drugs to specific patient populations, reduce the risk of drug side effects or drug-drug interactions, or assist in understanding negative results observed in clinical trials [73].

Kidney MPS could have applications in directly improving human health. For example, current hemodialysis techniques use one-way transport across a dialysis membrane to filter blood, which differs from a normally functioning nephroń s ability to filter and secrete toxins while reabsorbing necessary electrolytes and small molecules. This leads to the accumulation of compounds such as uremic toxins in dialysis patients [74]. A functional kidney MPS could, for example, provide an advancement to traditional hemodialysis treatment. There has been some previous success in improving AKI patient survival with bioengineered kidney devices [75].

Commercialization

Typically, MPS were engineered with the goal of modifying how drug candidates were discovered and assessed in pharmaceutical industry. Several companies have established their market in kidney-focused microfluidic devices. Microfabricated hydrogels and bioprinting integrated channel grids and endothelial cells to generate vascular networks, such as surrounding renal convoluted tubules [30,76,77]. Through this available technology, it allows the investigation of the 3D vascular-epithelial interface architecture and its impact on cellular and tissue functions.

Nortis Bio was derived from previous works at Washington University, which embedded a tubular microchannel inside a hydrogel as vascular and kidney models [29,78–80]. From Nortis, the ParVivo chips are populated with human organ-derived cells and inserted into a bioreactor and pneumatic pump to generate high-throughput models. Each generation of ParVivo safeguards a variety of configurations for organotypic cells types, concentration gradients, and multiorgan microenvironments. The MPS consists of casting an extracellular matrix around glass fibers that traverse a chamber within the chip. The fibers are extracted, leaving a perfusable channel, in which cells are seeded to form a tubular tissue [29,31,32]. These ParVivo chips have become integral in the Tissue Chips in Space initiative

to investigate how microgravity impacts human health and disease. Due to the rapid effects of space, tissue chips in space will simulate these effects, translating long duration on Earth [81,82].

Mimetas pioneered microfluidic hydrogel devices by applying a phase guide principle to control the patterning of hydrogels. Through this technique, a hydrogel-liquid interface can be assembled in a 384-well multiwell plate with gravity-driven flow, encouraging high throughput and rapid assessment in drug toxicology tests. Using this approach, Mimetas has demonstrated both glomerulus and proximal tubule capabilities. In OrganoPlate, glomerulus-on-a-chip was constituted with human podocytes and human glomerular endothelial cells (hGEC). The glomerular cells generated a layer of extracellular matrix composed by collagen IV trimer and laminin. In addition, glomerular filtration barrier was denoted based on its selective permeability and response to nephrotoxic compounds [23]. Similarly, within the OrganoPlate, 40 proximal tubules demonstrated real-time readout of loss barrier function, no artificial membranes, and functional transporter function [83–85].

Challenges

Although significant gains have been made in the advancement of kidney MPS technology, there is still work to be done. The human body is 3D, and 3D cell culture can improve the cell differentiation and function when compared with 2D culture [86]. Natural and synthetic hydrogels have been used in MPS models, including natural collagen, gelatin, alginate, and agarose, and synthetic polyethylene glycol and poly(lactic-*co*-glycolic acid) [10]. A 3D cell culture can more closely not only mimic the in vivo environment, but can also cause unwanted adsorption, limit nutrient and waste transport, and increase the difficulty of successful device operation. Multiple types of primary or stem cell-derived cells would best recreate kidney architecture and function, but finding a common cell culture medium that can support these different types of cells is challenging.

Data obtained with MPS must be extrapolated to predict drug efficacy and toxicity in humans. An exact mimic of a human tissue would provide the most authentic results, but that is difficult to achieve. Mathematical model adjustments can be made to account for differences between MPS and humans, including corrections for factors such as cell density, which is lower in MPS than the body; and cell enzyme/transporter functionality, which tends to be lower in vitro. Finally, in order to become a standard drug screening technique, kidney MPS need a standardized validation protocol for sensitivity and specificity (i.e., a sufficient and repeatable response to known nephrotoxic drugs and no response to compounds that do not target the kidney) [87] and to have accessible and reproducible operation.

Kidney MPS may surpass conventional preclinical testing models for predicting efficacy and toxicity of new compounds and potentially supplement human clinical trial data. These advances will provide a mechanism for directing resources toward the most promising new drug candidates. Although there are still significant challenges that remain in the field, continued research into kidney MPS development and validation has the potential for providing breakthroughs in drug discovery, disease modeling, and our understanding of kidney function.

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CHAPTER

26

Matrix scaffolds in kidney engineering

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Introduction

The extracellular matrix (ECM) is the 3D framework that makes the life of multicellular organisms possible [1]. It is secreted by resident cells of tissues and organs and consists in an interlocking meshwork of glycosaminoglycans, nidogen/entactin, and fibrous proteins like collagens, fibronectins, and laminins that provides a physical scaffold for maintaining the structural integrity of multicellular organisms [2]. Soluble signals, including cytokines, growth factors, and chemokines, are embedded within the ECM to be released or newly stored depending on the physiological needs and homeostasis of the local anatomical district. The ECM determines cellular behaviors, including cell survival, proliferation, migration, and differentiation, at any time during ontogenesis and afterward. Therefore, if we want to bioengineer cellular constructs that are able to exert a physiological function for clinical applications, we need to contemplate templates that replicate as much as possible the architecture, as well as the physical and chemical characteristics, of the ECM (Fig. 26.1).

This is valid also for the kidney ECM and for kidney bioengineering [3, 4]. The renal ECM is a very dynamic structure and different compartments show site-specific unique compositions of extracellular macromolecules designed to support the underlying functional needs of distinct nephron segments. Over two decades of research in kidney bioengineering have shown that ECM scaffolds can be successfully and consistently produced from virtually all species including humans, are completely acellular and virtually nonimmunogenic, maintain their architecture and essential molecular composition, lack cell membrane molecules, are able to determine cell phenotype and induce genes of renal development, possess remarkable angiogenic properties as demonstrated by the ability to induce vessel formation in the chorioallantoic membrane, are biocompatible in vitro and in vivo, and, when repopulated with renal cells, are able to show some function [2, 3, 5]. Moreover, when whole acellular porcine renal ECM scaffolds are implanted in pigs through a vascular anastomosis, the framework of the innate vasculature remains well preserved and is able to sustain physiological blood pressures [6].

In this chapter, we review the worldwide experience relative to the use of ECM scaffolds in renal bioengineering. The manuscript will span from whole organ bioengineering to the engineering of ECM-based biomaterials (mainly hydrogels) for the regenerative medicine approach to treat chronic kidney disease.

Whole organ bioengineering

The seminal paper from Doris Taylor's lab appeared in *Nat Med* in 2008 [7] represents a milestone in the history of regenerative medicine and organ transplantation, because it proposes for the first time whole ECM scaffolds as a valuable platform for whole organ bioengineering. Ever since, more than 1000 manuscripts have followed, showing that virtually all organs from all mammals may be decellularized to obtain ECM scaffolds that may be used as a

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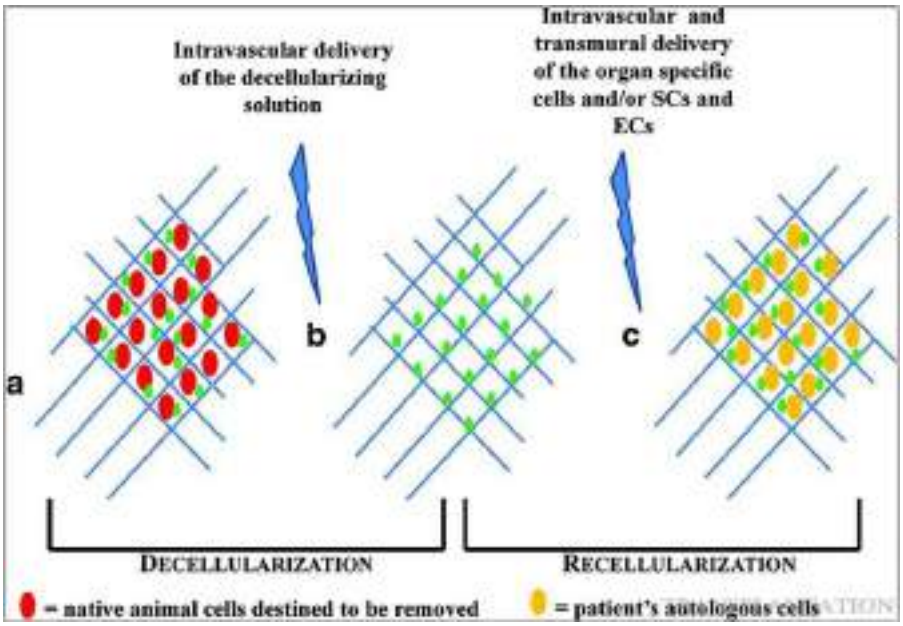
Intravascular delivery of the decellularizing solution
Intravascular and transmural delivery of the organ specific cells and/or SCs and ECs
a
b
c
DECELLULARIZATION
RECELLULARIZATION
= native animal cells destined to be removed
= patient's autologous cells

FIG. 26.1 Schematic of decellularization. The organ's cellular compartment is cleared through the intravascular injection of a decellularizing solution, which normally contains various detergents. The acellular organ is therefore produced and will be used as template for the regeneration of the missing cellular compartment, ideally using patient's own cells. This will render any sort of antirejection medication unneeded after implantation of the so-bioengineered organ. *From Orlando G, et al. Regenerative medicine and organ transplantation: past, present, and future. Transplantation 2011;91:1310–17. https://doi.org/10.1097/TP.0b013e318219ebb5, with permission.*

template for organ bioengineering. This section will summarize the experience with using renal ECM as a template for the bioengineering of functioning kidneys. This will be done by briefly illustrating the results obtained by the most representative authors in the field.

Rodent models

In 2009, Ross et al. were the first in reporting that the infusion of a detergent-based solution throughout the vascular pedicle allows the complete clearance of the cellular compartment of rat kidneys, while the innate ECM structure and architecture remain quite preserved [8]. The decellularization protocol (Fig. 26.1) was based on 5 days of continuous perfusion with ionic and nonionic detergents, sodium chloride, deionized water, and DNase through the renal artery at a pressure of about 100 mmHg. The measures of outcome evaluated by authors were the complete decellularization of the native kidney in combination with the preservation of the 3D framework of the ECM, as well as of its basic components like collagen IV and laminin. This was assessed with some straightforward histological tests which confirmed the absence of any cell or cell debris, while serial staining showed the presence of a solid network of collagen and laminin fibers. No information about the quantitation of DNA, collagen, laminin, elastin, or glycosaminoglycan (GAG) after decellularization was provided, and it was not determined whether any detergent remained within the acellular scaffolds. Moreover, authors seeded scaffolds with embryonic stem cells, by direct anterograde injection through the artery and direct retrograde injection through the ureter. Interestingly, primitive precursor cells attached, populated and proliferated ubiquitously within the glomerular, vascular, and tubular structures. Very importantly, these authors made the fundamental observation that these cells had changed their phenotype by the end of the first week. In fact, the evidence that the ECM of a given organ is able to induce the expression of genes critical for the development of the organ in question is of paramount relevance in regenerative medicine and is consistent with Bissell's early intuition of the "dynamic reciprocity" between the ECM on the one hand and the cytoskeleton and the nuclear matrix on the other hand, that represents one of the milestones in the history of ECM biology [9]. On the wake of these successful preliminary investigations, in 2012 the same group provided evidence that pluripotent precursor cells seeded into rat renal ECM acellular scaffold vasculature differentiate into endothelial cells, which in turn follow a normal ontogeny as they remodel the laminin and collagen of their basement membranes [10]. These authors first tested vascular cell endothelialization by endothelial-specific BsLB4 lectin and anti-VEGFR2 (Flk1) antibodies; then, the remodeling of the matrix basement membranes from rat to mouse (referred to as "murinization") was assessed by a monoclonal antibody specific for mouse laminin β1 chain. Later, in an elegant study

where biocompatible acellular ECM scaffolds could be obtained through continuous infusion of SDS (sodium dodecyl sulfate) into rat kidneys for only 17 h, Remuzzi's group observed the loss of cell pluripotency and the start of differentiation toward the meso-endodermal lineage [11].

This work paved the way for different protocols to optimize the decellularization process, as well as to reconstitute the parenchymal and vascular compartment of the kidney in the rat model. In 2013, the Ott's lab reported the successful seeding of acellular rat scaffolds with rat neonatal kidney cells to reconstitute the parenchymal cell compartment, and with human umbilical venous endothelial cells to reconstitute the endothelium and allow implantation [5]. These authors combined antegrade seeding of endothelial cells with retrograde seeding of the kidney cells under the application of a transrenal pressure gradient in an ad hoc designed bioreactor. Cells were able to spread throughout the 3D framework of the scaffold, repopulate half of the glomeruli, and could be found in different segments of the nephrons across the scaffolds while expressing tissue-specific markers. Furthermore, the partially recellularized kidney scaffolds were assessed for function both ex vivo and in vivo. Ex vivo, authors demonstrated some degree of filtration capacity when compared to acellular ECM scaffolds and cadaveric kidneys; moreover, parameters including vascular resistance, albumin retention, and glucose reabsorption were lower in the regenerated kidneys when compared with intact kidneys procured from dead rats. In vivo, the group implanted the regenerated kidneys orthotopically into recipient rats and showed that urine-like solution could be produced although at lower levels than in native kidneys; furthermore, creatinine, urea, and albuminuria were lower than in native kidneys but higher than in decellularized kidneys in control animals. This study, just like Ross's first paper, represents a milestone in the literature, which is of interest for the following three reasons: first, because for the first time it reported on the development of a dedicated technology that may be applied to regenerate—or bioengineer—both cellular compartments (endothelium and parenchymal) of the kidney; second, because for the first time, authors very bravely attempted to address the question of how to restore the function of renal organoids both in vitro and in vivo; third, because it emphasized the role of bioreactors as well as of seeding strategies aimed at reconstituting the cellular compartment.

Function was studied by the group at Shandong University, China, which also assessed the amount of critical growth factors as a measure of outcome of their decellularization method [12]. Wertheim's group evaluated growth factors as well, while attempting the optimization and critical evaluation of decellularization strategies to develop renal extracellular matrix scaffolds as biological templates for organ engineering and transplantation [13]. Moreover, for the first time his group reported on the use of human iPSC-derived endothelial cells and tubular epithelial cells to repopulate their biomatrix obtained with Triton/SDS-based solution.

Porcine models

Pigs are the most common animal source of clinical xenografts and represent the ideal model for clinically relevant investigations in the field of kidney—and, more broadly, organ—bioengineering and regeneration. In the attempt to identify a new potentially inexhaustible source of organs for transplant purposes, the pig may represent that invaluable source both as a source for xenografts, as well as a source for ECM scaffolds [14, 15]. In 2012, our group at Wake Forest reported for the first time the use of porcine kidneys as a source of renal ECM scaffolds for kidney bioengineering investigations [6]. We showed that ECM scaffolds can be successfully and consistently produced from the porcine kidney. Such scaffolds are acellular, maintain their 3D architecture and molecular composition, show an intact and patent innate vascular tree, and are biocompatible both in vitro and in vivo. In fact, when cells are seeded on fragments of ECM, they attach and remain viable for 1 week. When acellular scaffolds are implanted in pigs and kept in situ for 2 weeks, animals tolerate the scaffold well. Importantly, for the first time evidence was provided that acellular scaffolds are able to sustain physiological blood pressure despite the absence of muscle cells and endothelium in the vasculature. Moreover, it was also noticed that the framework of the innate vasculature is resilient and able to respond to modifications of the applied pressure, when the scaffold is perfused with solutions. This information is of critical importance in view of translation, and suggests that ECM-based scaffolds may be the scaffold of choice in organ bioengineering. In fact, synthetic scaffolds lack the innate vasculature, and no technology is currently capable of reproducing the vascular network of any given tissue or organ. Of note, when porcine acellular scaffolds were procured and analyzed with H&E staining, as expected, the whole vasculature was filled with thrombi due to the lack of endothelium. Later on, the same group from Wake Forest published a study showing that 0.5% SDS was the most effective solution for decellularizing porcine kidneys [16].

In an attempt to develop strategies to recellularize acellular ECM scaffolds, it is important to understand how the ECM interacts with cells. To address this fundamental question, O'Neill et al. derived a method to develop region-specific ECM biomaterials (namely, ECM sheets, ECM hydrogels, and solubilized ECM) for stem cell culture from the three regions of the porcine kidney—cortex, medulla, papilla [17]. The objective of the study was to

determine if there were region-specific effects of kidney ECM on the growth and metabolism of kidney-derived stem cells (KSC), how these effects depend on the preservation of ECM structure versus composition alone, and if these effects extend to exogenous (nonkidney) stem cells, such as mesenchymal stem cells (MSCs). Interestingly, when KSC were seeded on renal ECM and control ECM from other organs, it was found that ECM biomaterials derived from the kidney affect the growth and metabolism of KSC with regional specificity. In particular, there is a significant degree of recognition and specificity between adult kidney stem cells and their extracellular environment. Stem cells showed significantly higher proliferation and higher metabolic activity in kidney ECM when compared to KSCs in ECM from other organs. In addition, KSCs showed lower proliferation and higher metabolic activity when cultured in papilla ECM (kidney stem cell niche) compared to medulla and cortex ECM.

Guan et al. reported a new method for repopulating ECM renal scaffolds, consisting of multiple injections with a 28G needle throughout the porcine kidney [12]. Another group demonstrated the feasibility of seeding porcine primary renal cells into whole-renal porcine ECM scaffolds by multiple injections with 23G needles, after preconditioning of the scaffold prior to seeding by 1-day flushing with a specific culture medium [18]. Briefly, renal cells were suspended in media at a concentration of 10×10^6 cells/mL and then delivered into the cortical region of the renal scaffolds using a 23G needle. The injections started in the periphery of the kidney in the upper pole. The distance between the different injection sites was 2.5 mm with a depth of 5 mm. 0.5 mL (5 million cells) of the cell suspension was injected at each site of both the anterior and posterior surfaces of the upper pole of the kidney. In total, 400×10^6 cells were injected into each kidney, and, at the completion of the seeding process, the seeded scaffolds were incubated statically for 30 min before being subjected to perfusion culture for up to 28 days. During the study, the organoid seemed to be viable and exert function. Reportedly, this method grants repopulation of approximately 40%–50% of the upper pole of the kidney. The same group also attempted the regeneration of the endothelium using antibodies to bound endothelial cells that were conjugated to the ECM in order to improve retention of endothelial cells.

As the detergents currently used to obtain ECM scaffolds could be damaging to the microstructure of the renal tissue and may undesirably solubilize the endogenous growth and signaling factors, some authors have tried to optimize the method in order to minimize the damage to the ECM. The group in Provo, United States, recently illustrated an automated decellularization method for porcine kidneys combining physical and chemical steps [19]. Briefly, freezing/thawing, incremental increases in flow rate under constant pressure, applying osmotic shock to the cellular membranes, and low concentrations of SDS were used to decrease SDS exposure time during the decellularization process from 36 to 5 h, which preserved the microstructure while still removing 99% of the DNA. Importantly, GAGs were almost totally preserved, leading to an enhancement of cell-ECM interactions. Human renal cortical tubular epithelium cells grew more rapidly when cultured on the ECM obtained from the improved decellularization process and also demonstrated more in vivo-like gene expression patterns. Notably, the degree of GAG's preservation represents a critical metric to assess the accuracy of any decellularization method [20].

Nonhuman primate models

Rhesus monkey kidneys have been proposed by Tarantal's group as an ideal platform for investigations aiming at assessing the role of ECM scaffold in the field of kidney regeneration, repair, and bioengineering. In their seminal studies, authors have demonstrated that decellularized sections of rhesus monkey kidneys of all age groups provide a natural ECM with sufficient structural properties with spatial and organizational influences on human embryonic stem cell migration and differentiation and that the age of the donor is a critical factor in repopulation efficiency [21–25]. Interestingly, ECM allows the formation of nephron structures, stores several residual growth factors that may be beneficial in scaffold recellularization, and retains antimicrobial proteins that may be beneficial for in vitro culture as well as downstream host integration.

ECM scaffolds derived from the human kidney

It is envisioned that, when this technology will be able to be translated to the bedside, a patient needing a new kidney will receive a brand new kidney manufactured from his own "biomaterials." In fact, the patient will be subjected to the robotic removal on one of the native kidneys that will be thereafter processed in order to obtain healthy cells, as well as an acellular scaffold that will serve as template for the bioengineering of his or her new kidney. This vision has justified the use of discarded kidneys as a platform for kidney bioengineering [26, 27]. For instance: only in the United States, up to 2600 kidneys that were initially procured for transplant purposes are eventually discarded due to poor biopsy, surgical damage, long preservation, etc. [28] Our group was the first to conceptualize the use of human discarded kidney as a source of ECM scaffolds for kidney bioengineering, and to show that also the

human kidney can be decellularized to produce ECM-based scaffold to be used as template for the construction on a transplantable kidney [3, 27–29]. Investigations showed that detergent-based decellularization yields acellular ECM scaffolds, as in the case of the rodent, porcine and nonhuman primate models. Notably, the most relevant difference between human and nonhuman ECM scaffolds is that human scaffolds consist of diseased or damaged matrix, which raises concerns about its usefulness. However, as evidence of fibrosis reversibility has been provided in a myriad of clinical settings, the use of discarded kidneys has become increasingly popular in tissue engineering, as demonstrated by the plethora of primary papers on the topic released in the past 5 years. In the case of the human kidney, this work has been fundamental in showing that the ECM intrinsic to human organs seems to be an extremely useful biomaterial for whole organ bioengineering for several reasons, among which is the evidence that they maintain the framework of the innate vasculature which is critical for implantation in vivo. Preliminary investigations have shown that ECM scaffolds can be successfully and consistently produced from the human kidney, and display the following key features: they maintain their architecture; their gross molecular composition and vasculature are preserved; they lack immunogenic cell membrane proteins; and they retain numerous bioactive growth factors that are stored in the 3D structure of the ECM, as demonstrated by the fact that they induce angiogenesis in a chorioallantoic membrane assay [3, 28]. Importantly, when multipotent stem cells are seeded on such scaffolds, cells attach well, are able to migrate throughout the 3D framework of the scaffold, remodel the ECM, and show the ability to mount an inflammatory response and promote angiogenesis [29].

This notwithstanding, experience specific to the kidney ECM but also—and more broadly—to the application of decellularization technology to a myriad of mammal tissues including the organs that are currently transplanted (namely, livers, hearts, lungs, small bowel, pancreas, and vascularized composite tissues), shows that clinical translation will not occur within the next decade. In fact, since Ott's seminal report [7], more than 1000 manuscripts have followed, showing that any mammal tissue or organ can be decellularized to obtain ECM scaffolds that may be used as template for tissue or organ bioengineering; however, no author has ever reported the implantation in vivo of a viable and functioning decellularized-recellularized organ. Table 26.1 summarizes the numerous hurdles to overcome before this technology may be translated to the bedside. Some of them are applicable to all organs while others are organ-specific (see, e.g., the need to nerve regeneration in the case of vascularized composite tissues and organs like hands, limbs, or the face and face subunits).

Briefly, the following milestones need to be reached on the way to the translation of this technology (Fig. 26.2):

(a) First, we need to better understand how the human kidney develops, as well as the mechanisms with which the kidney repairs itself after damage. Worldwide, kidney development has polarized the attention of countless research teams as well as of funding institutions and agencies. However, due to the inherent difficulties associated

TABLE 26.1 Synoptic view of the hurdles to overcome before organ decellularization can be translated into the clinic.

Area	Focus for future research
General	Better understanding of ECM-cell interactions and physiology Better understanding of organ ontogenesis Scale up
Biomaterials	Combinations to achieve desired functional, mechanical, and supportive properties ECM
Cell sources	Most tissues require multiple cell types Need improved methods to differentiate stem cells into *all* required cell types Cancer degeneration
Vascularization	Well-developed vascular tree required for large tissues May have to be engineered in the bioprinted construct Capillaries and microvessels required for tissue perfusion Suitable mechanical properties to sustain physiological pressures
Innervation	Innervation is required for normal tissue function May be inducible after transplantation using pharmacological or growth factor signaling Simulation before transplantation could be achieved using bioreactors
Maturation	Time required for assembly and maturation Bioreactors may be used to maintain tissues in vitro Provide maturation factors as well as physiological stressors Potential for preimplantation testing of constructs

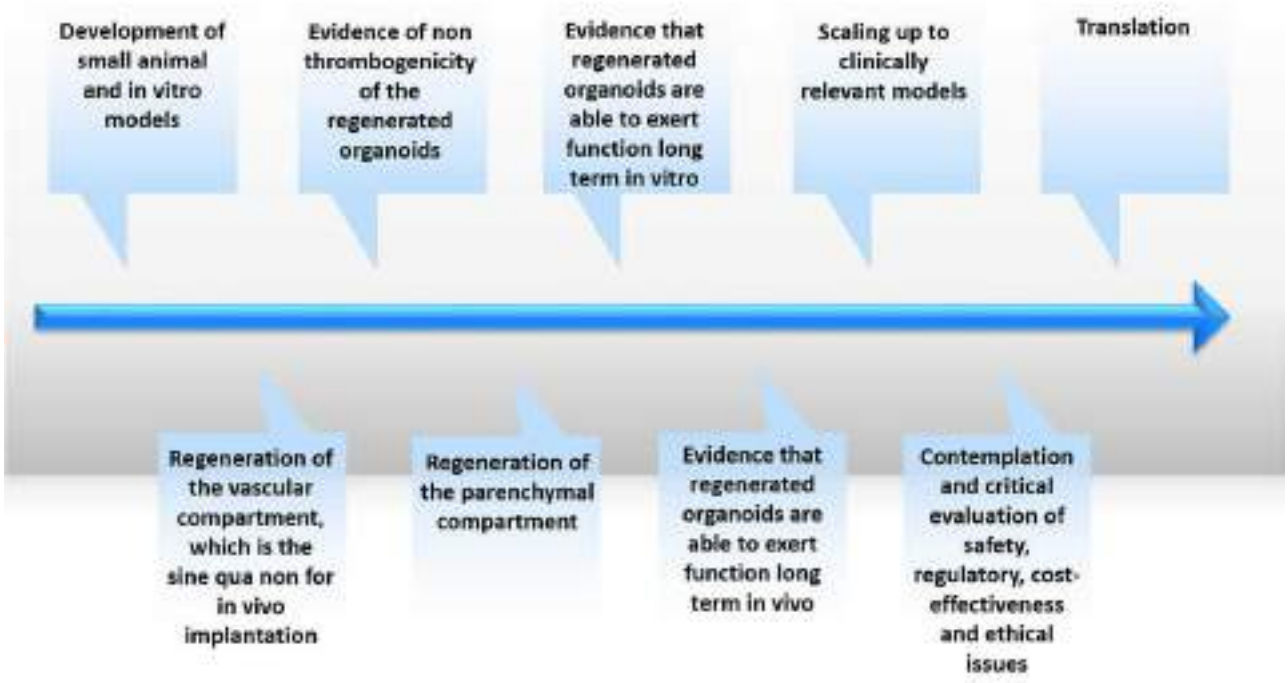
Development of small animal and in vitro models
Evidence of non thrombogenicity of the regenerated organoids
Evidence that regenerated organoids are able to exert function long term in vitro
Scaling up to clinically relevant models
Translation
Regeneration of the vascular compartment, which is the sine qua non for in vivo implantation
Regeneration of the parenchymal compartment
Evidence that regenerated organoids are able to exert function long term in vivo
Contemplation and critical evaluation of safety, regulatory, cost-effectiveness and ethical issues

FIG. 26.2 Roadmap for translation of decellularization and 3D printing technologies. *From Orlando G, et al. Rethinking regenerative medicine from a transplant perspective (and vice versa). Transplantation 2019;103:237–49. https://doi.org/10.1097/TP.0000000000002370, with permission.*

with investigating the development of human organs, our knowledge of kidney development is based mainly on small animal models. On the other hand, investigations aiming at revealing the mechanisms through which the kidney repairs itself are often conducted on patients who are experiencing different degrees of renal impairment, so bypassing the biases that may derive from nonhuman models.

(b) Second, when the ex vivo engineering (synonymous with fabrication) of a transplantable kidney organoid is considered, current investigations are being conducted without any criteria *vis a' vis* of the nutrients, oxygen, and energy supply required by the new organ to develop. There is basically no information about how much oxygen, nutrients, and energy a developing kidney needs to complete its maturation and be considered viable. Indeed, a critical step will be to achieve the maturation of the seeded scaffolds in custom-made bioreactors, yet long-term maturation that recapitulates kidney ontogenesis (up to 3 months) has never been reported and is burdened by a high risk of super infection of the bio-system.

(c) Third, reports on the characterization of the different renal ECM scaffolds are scattered, measures of outcome are inconsistent, and a unifying vision is missing. Therefore, it will be of paramount importance for investigators in the field to unite forces, share experiences and combine assets, specializations, and visions.

(d) Fourth, strategies to recellularize ECM scaffolds are still at their early stages, and results are far too preliminary. Moreover, scalability to more clinically relevant models seems hard at the moment.

(e) Fifth, it remains unclear which cells should be used—progenitor cells with different degrees of stemness, adult cells, iPS, etc.—to repopulate acellular scaffolds in order to regenerate the parenchymal and vascular compartments. While progenitor cells have a huge potential given their ability to generate different cell lineages, the risk of tumourigenesis cannot be ruled out and represents a significant burden even if investigations are still far from any clinical applicability. Even in this case, coordination and integration of ad hoc research is desirable and would certainly foster progress.

(f) Sixth, current technology lacks appropriate and efficient bioreactors and mathematical models.

However, a consideration should be made: the decellularization approach to organ (and therefore kidney) bio-engineering is a *top-to-the-bottom* approach whereby the organ that needs to be bioengineering is subjected to the decellularization process. The organ is "compatible" size-wise and anatomy-wise to the patient in need for the new organ, but will have to be subjected to a process (the decellularization itself) that will somehow alter the anatomical

characteristics of the ECM. Instead, we should probably consider a *bottom-up* approach, whereby we start from the patient to then bioengineer the organ in question. This will be made possible using a combinatorial approach whereby 3D printing technology and artificial intelligence are harnessed. 3D printing in fact is a form of additive manufacturing where objects (in our case, organs) are manufactured in layers. In our specific case, a patient in need of a new kidney will be imaging-scanned to obtain information of the "needed" anatomy. This information will be analyzed by the software of the 3D system in conjunction with the artificial intelligence that will eventually determine the required anatomy and histology, as well as what will be required—in terms of cells and ECM—to manufacture every single layer of the desired organ (see cartoon).

Renal ECM hydrogels

Hydrogels are highly hydrophilic polymeric materials that swell in contact with water and are formed by crosslinks between polymer chains based on either noncovalent (i.e., physical) or covalent (i.e., chemical) binding, or a combination of both types [30]. They are among the most popular biomaterials in modern tissue engineering and offer the quickest route to clinical translation. Due to their polymeric nature, hydrogels are highly tunable in terms of mechanical properties. By changing system parameters like crosslinking density or polymer concentration, stiffness can be adjusted to better represent a specific type of tissue. The strategy of crosslinking reactive hydrophilic polymer solutions into hydrogels allows formation into different geometries, like microspheres, tubes, or nanofibers. Moreover, composites with other hydrogels or nonhydrogel materials can be used either as de novo scaffolds or to reinforce existing scaffolds.

Kidney-derived ECM hydrogels have been developed and investigated in the past few decades and evidence has been provided that they are able to support cell attachment, viability, and proliferation [31]. Interestingly, a large body of literature shows that region-specific kidney-derived ECM hydrogel are able to modulate cell response and may maintain an immature, stem cell-like phenotype [32] or a more mature, quiescent phenotype [33]. In the first case, O'Neill et al. demonstrated that kidney stem cells adopted a significantly more proliferative and metabolically active phenotype when cultured in the presence of kidney ECM hydrogels as opposed to heart or bladder ECM [32]. Furthermore, they also demonstrated that kidney stem cells cultured in the presence of ECM hydrogels obtained from the renal papilla resulted in lower cell proliferation, higher metabolic activity, and altered cell morphology in comparison to cortex- and medulla-derived ECM. This finding was of interest because, as the renal papilla represents the native kidney stem cell niche, it could be inferred that any biomaterial derived from the papilla—including the therein described hydrogel—would maintain a stem cell phenotype as suggested by their findings. Moreover, the authors propose that cortical- and medullary-derived ECM may show potential in directing the differentiation of kidney stem cells toward specific renal lineages.

In the second case, Nagao et al. examined the influence of human kidney cortex-derived ECM hydrogels on human a very specific cell line, namely kidney peritubular microvascular endothelial cell (KPMEC) [33]. When cultured on the above-mentioned hydrogels (study group) and collagen-I (control group), these cells showed an upregulated gene expression of molecular signatures suggestive of cellular quiescence and maturation; at the same time, they featured a concomitantly decreased expression of genes associated with proteolytic activity and cell surface activation, when compared to control group. Moreover, when HUVECs were used, these latter appeared more quiescent on collagen-I and, contrarily to KPMEC, more stimulated on the cortex-derived ECM hydrogels.

Other groups are contributing to the progress of the field [31, 34, 35], While it is quite apparent that, for accurate mimicry of a physiological setting, hydrogel mixtures with solubilized tissue-specific ECM components appear to hold the greatest potential, it should be noted, however, that the use of biologically derived materials is accompanied by the risk of contamination with residual cellular debris or endotoxins, quick degradation or contraction, and batch-to-batch variabilities [30]. Moreover, hydrogels will have to meet application-specific requirements, such as suitable viscosity, overall charge, biodegradability, and mechanical properties. This notwithstanding, ECM-based hydrogels hold high potential for clinical translation also in form of bio-ink for renal tissue bioprinting [36] or cell delivery tool to mitigate renal ischemia-reperfusion injury [37].

Bio-printing scaffolds

Perfusion-based decellularization uses a top-down approach taking a native organ and making an acellular ECM bio-scaffold that maintains the renal, vasculature tubules, and lymphatic system. An alternative approached for whole organ scaffold creation is 3D bio-printing, as mentioned above. Bio-printing is a bottom-up approach that

focuses on select components (e.g., renal tubule) of the organ and utilizes advanced technology to print these components in the shape of the desired organ or scaffold. Printers function similar to a printer at home, cells and bio-ink are printed in a 2D fashion and once layered produce a 3D structure. Templates and binders help give rise to the desired shape and construct of the biomaterial during the printing process.

An advantage of bioprinting is the ability of the bio-ink to protect cells from damage. However, the biocompatibility of bio-inks is limited and bioprinting is in the early stages of development. Printing complex organs such as the kidney is only in the early developmental stages with current technology. Some problems of bioprinting a kidney are:

(1) Complex cellular composition—the kidney contains over 20 different mature cell types that have specific functions regulating excretion, filtration, secretion, and urine production. Bio-printing different types of cells and directing them to a specific location at the same time all at once is not yet possible.
(2) Chemical and biochemical microenvironment/biotechnological limitation—like all organs the kidney has its own microenvironment composed of proteins, growth factors, immune mediators, and membrane exchange and transport systems. Most bio-inks do not support all of these functions. A bio-ink is a substance that is made by a living cell and provides structured support mimicking the ECM. Bio-ink supports adhesion, proliferation, and differentiation of cells and is fluid enough to be printed by a printer. Current research efforts aim to broaden the scope of bio-inks and optimize them for specific chemical environments.
(3) Architecture/technological limitation—The kidney is composed of durable and elastic ECM structures that support tubular formation. These vascular and nephron tubules are convoluted, contain countercurrent exchange membranes, and have a dynamic flexible organization. Vasculature and nephron tubules are microscopic in size ranging from ~ 200 μm to as small as 50 μm in diameter. In order to print such fine tubules and dynamic structures, the printer must be able to print multiple components at once, analyze multiple images that range in scale, and the print head must extrude bio-ink based on the scale with the procession.

A myriad of biological and mechanical factors needs to be optimized before bio-printing organs as complex as the kidney becomes feasible. But bioprinting holds the immense potential to change the way we approach tissue and organ transplantation.

Bio-inks

The preferable biomaterial that provides fluidity and rigidity under applied conditions is a hydrogel. As briefly illustrated earlier, hydrogels can retain water and form a 3D network of polymers that either mimic ECM properties or is an ECM-based hydrogel. Artificial and natural hydrogels have been demonstrated to be biocompatible without producing an immunogenic response and rejection of the gel. Artificial hydrogels are generally crosslinked, noncovalent, or covalent bond formations that are molded to the desired network or support system. Generally, this process takes a liquified precursor that once activated forms a rigid, yet elastic structure. The elasticity of the structure is dependent on the concentration of the polymer, temperature, time, and pressure during the cross-linkage process.

Type 1 collagen, hyaluronic acid (HA), chondroitin sulfate (CS), fibrin, and solubilized ECM can all be used as bio-inks.

(i) The most basic of natural bio-printable ink is type I collagen. Type I collagen is a liquid in both basic and acidic conditions, but upon neutral pH conditions can crosslink to form a semirigid structure. Collagen is the basis of ECM composition providing rigidity and structural support to the ECM, thus collagen type I is biocompatible, maintains high cell viability, and was one of the first bio-inks implemented as a therapeutic device [38].
(ii) HA is another commonly employed biomaterial used as a bio-ink. HA is used with other photo-crosslinkers or photo-crosslink initiators due to its structural integrity after cross-linkage. HA has been shown to be biocompatible making it a successful hydrogel scaffold with the ability to release growth factors over time. HA also has high cell viability, but not as high as collagen type I. Dextrin, chondroitin sulfate, and gelatin are frequently combined with HA to improve its shape and flexibility once crosslinked [39, 40].
(iii) CS has poor rigidity and shape upon cross-linkage. Therefore, polyethylene glycol (PEG) or other chemical crosslinkers are added to chondroitin sulfate in order to enhance the biocompatibility, rigidity, and elasticity of the hydrogel scaffold produced [38].
(iv) Fibrin is suitable as a bio-ink that provides a stiff and biocompatible environment. Fibrin allows cells to produce their own ECM when the right growth factors are added [41–43]. Fibrin also helps with cell proliferation and enables cells to adapt the physical environment to fit their shape and need. Advantages of fibrin are its ability

to be used in printing microsystems including small vessels and tubules. The disadvantage of fibrin is its transparency and gelatinization. Gelatinization is easily controllable, induced by calcium or the presence of thrombin.

(v) Solubilized ECM is one of the most important and popular natural substances used as a bio-ink because it provides the biochemical environment needed for cells to grow and mature, which cannot be completely provided by the above bio-inks. ECM also does not require the addition of potentially toxic chemicals to induce cross-linkage. Current research efforts are focused on producing a printable ECM-based ink that has the appropriate fluidity and flexibility and upon cross-linkage is supportive and rigid [36]. Solubilized ECM is fluid enough to be printed, however, viscous enough to protect cells during the printing process. One group of investigators has proposed soluble kidney ECM and powdered ECM as a bio-ink for 3D kidney bio-fabrication [44]. Future studies will focus on solubilized ECM as a bio-ink printing 2-dimensional (2D) scaffold that can be seeded with cells and/or implanted in surgery to provide a therapeutic platform for cellularization.

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PART VI

Emerging clinical aspects of regenerative therapy

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CHAPTER

27

Reprogramming toward kidney regeneration: New technologies and future promises

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Introduction

The ultimate goal of regenerative medicine is to restore organ or tissue function by replacing the damaged or lost cells. The classic regenerative approach involves the transplantation of cells, generated or expanded in vitro, into the damaged organ or tissue. Until today, the most successful example of organ regeneration is bone marrow transplantation, which remains the standard clinical practice for regenerating the human hematopoietic system. Unfortunately, not all organs possess a well-defined stem cell population that can replenish the entire cell repertoire like hematopoietic stem cells (HSCs). Hence, transplantation typically requires an entire donor organ such as a kidney or a substantial amount of a donor organ such as a liver, either from a cadaver or from a live donor. Furthermore, living with an organ transplant, in most cases, requires the recipient to continually take immunosuppressors, which may cause additional health complications due to a decreased activity of the patient's immune system. The increase in the global incidence of end-stage renal disease and the burgeoning demand for transplantable kidneys have made it crystal clear that novel regenerative therapeutics are needed to help alleviate kidney disease and associated health issues.

The mammalian kidney is regarded as a nonregenerative organ, since no new nephron forms after birth. Each individual is born with a specific number of nephrons that will gradually decline as we age. The only possible way to replenish lost nephrons is to introduce exogenous ones, which is traditionally achieved via kidney transplantation. Nonetheless, it has been known for decades that the kidney has a potent cellular regeneration capability, which can restore the damaged tubular epithelial cells, in response to acute injury. Although the mechanism of kidney cellular regeneration has not been explicitly elucidated, cell plasticity is regarded as a central player in this critical process [1]. However, this intrinsic capability may reach an upper limit in some extreme cases. Consequently, once the damage is beyond the levels that can be fully attended by the intrinsic cellular regeneration of the kidney, the damaged tissue will undergo some undesirable pathological pathways that eventually lead to chronic kidney diseases and renal failure [2]. Although we are still far away from developing optimal approaches for kidney regeneration, remarkable progress has been made during the last decade.

Cell plasticity

The rate-limiting step of regenerative medicine is the ability to obtain the desired cells that possess matching properties and quantities for restoring the target organ. Ideally, these cells could be produced by the injured organ itself, obviating the necessity for transplanting exogenous cells. Regrettably, during evolution, humans have been deprived of the spectacular regenerative capability seen in other species, such as Axolotls, which can regenerate an entirely lost limb [3]. Despite the importance in maintaining cellular identity for normal tissue function throughout an organism's lifespan, most cells can change their identity, a phenomenon known as cell plasticity, which has

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long fascinated biologists. In both experimental and natural settings, cellular identity can be altered. The most pronounced example was shown by John Gurdon that mature adult cells can be converted into a fertilized egg-like state by nuclear transfer [4].

Cell fate conversion can be broadly categorized into two types: dedifferentiation and transdifferentiation [5]. Dedifferentiation refers to the reversion of a differentiated cell to an earlier stem/progenitor state within the same lineage during development. Taking gastric epithelium for example, a small subpopulation of fully differentiated gastric chief cells expressing Troy can give rise to the entire spectrum of epithelial lineages of the gastric corpus [6]. The Troy$^+$ chief cells represent a reserve stem cell population that can be stimulated to dedifferentiate upon injury, contributing to repair [6]. The intestines possess a defined resident stem cell population marked by Lgr5. When extreme crypt injury eliminates all Lgr5$^+$ intestine stem cells, almost all intestine epithelial lineages (enterocytes, enteroendocrine cells, secretory progenitors, and transit-amplifying cells) can be dedifferentiated into Lgr5$^+$ stem cells to attend to the injury [7–11]. Likewise, epidermis [12, 13], neuronal epithelium [14], and airway epithelium [15] adopt a similar, albeit organ-distinctive approach for regeneration.

Apart from dedifferentiation, transdifferentiation (direct cell fate conversion), in rare cases, also takes place to restore the injured organ in a natural setting. When adult mice encountered massive beta-cell loss in the presence of insulin injection, alpha-cells can transdifferentiate into beta-cells to restore glycemic control [16]. In another scenario, when similar beta-cell loss occurs in young mice, somatostatin-producing delta-cells are reprogrammed into beta cells in vivo [17]. Interestingly, this delta-beta cell transdifferentiation proceeds through a less-differentiated intermediate cell state.

Within an organism, cellular identity is maintained through epigenetic regulation, resulting in heritable patterns of tissue-specific gene expression. Historically, the Waddington epigenetic landscape has been employed to conceptualize the developmental choice made by tissues during embryonic development. The increasing body of evidence has considerably revised the traditional Waddington diagram by adding regenerative dedifferentiation and transdifferentiation routes that do not always follow the developmental paradigm (Fig. 27.1) [18]. The establishment of an epigenetic landscape for kidney regeneration will have important clinical implications by revealing and predicting the regenerative cell types and pathways [1].

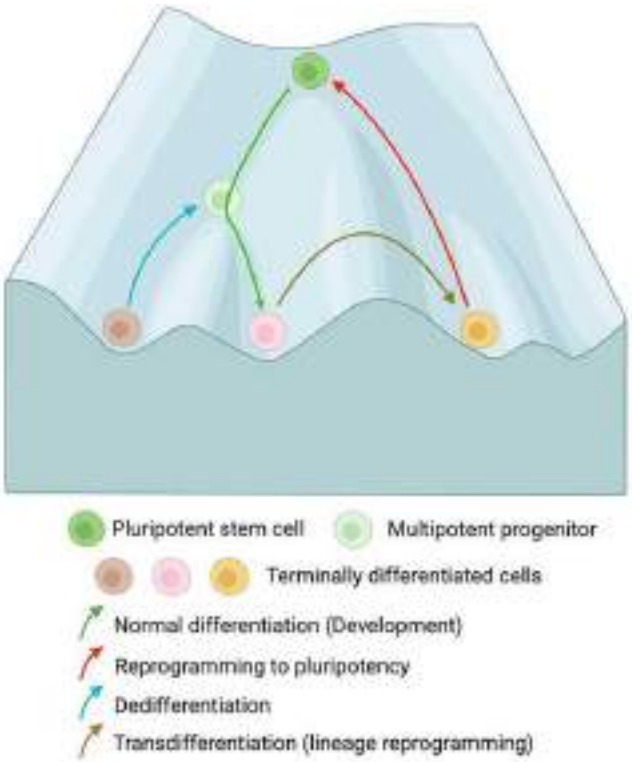

FIG. 27.1 Revised Waddington epigenetic landscape demonstrating cell plasticity.

Induced pluripotent stem cells—An extreme example of cell plasticity

The conversion of terminally differentiated somatic cells into a pluripotent-like state via forced expression of pluripotency factors represents the most extraordinary example of cell fate plasticity [19]. The establishment of the reprogramming approach has made it possible for us to derive, in theory, any cell type from an individual's own cells via a detour to induced pluripotent stem cells (iPSCs) [20]. Since then, there has been a collective effort to formulate differentiation recipes to generate a spectrum of cell types from human pluripotent stem cells (PSCs) for understanding human disease, testing drugs, and most importantly, restoring damaged organs. The last 10 years have borne witness to a large body of studies that aim to steer human PSCs into miniature organs (organoids) that present both the cellular repertoire and three-dimensional (3D) structure of the cognate organ [21, 22]. Despite the remarkable success in recapitulating critical structural and functional features in human PSC-derived organoids, they are far from being suitable for clinical use due to immaturity, limited dimension, variability, and a lack of structural and functional sophistication [23]. The development of novel tools is bringing us closer to building organoids that better resemble their in vivo counterparts, in a step-wise manner. Single-cell analysis reveals organoids' cellular composition and their respective features, allowing us to bench mark organoids against their cognate organs. CRISPR-Cas9 gene editing tools make it feasible to introduce genetic markers for enriching desired populations. There is growing evidence that the physical environment imparts a nontrivial influence on organoid phenotypes. Bioengineering tools, such as microfluidics and synthetic hydrogels are being actively engaged for recapitulating physical clues that are missing in traditional differentiation culture. Much remains to be done before we change stem cell-based replacement therapy from theory into clinical practice.

In vitro nephrogenesis I—A detour via iPSCs

Direct differentiation to specific renal lineages

In 2005, 1 year prior to the debut of iPSCs, a landmark study by Dressler and Kim demonstrated successful differentiation of mouse embryonic stem cell (ESC)-derived embryonic body (EB) into renal epithelial cells that were able to self-organize into 3D structures via spinal cord co-culture or upon injection into mouse embryonic kidney rudiments [24]. A few similar studies have also nudged mouse ESCs toward renal epithelial lineages, primarily through influencing spontaneous ESC differentiation using either a few renal growth factors [25, 26] or through engaging embryonic kidney co-culture [27]. These pioneering studies, though intriguing, generally lack a thorough and meticulous control of the differentiation process.

Alongside the establishment of the iPSC reprogramming methodology in 2006 [19], our understanding of kidney development was also advanced to the next level, owing to the identification of a $Six2^+$ self-renewing nephron progenitor population during kidney development [28]. Due to improvements in human iPSC reprogramming and culture platform in the following years, several better-defined differentiation protocols were established for generating specific types of renal cells from mouse and human PSCs, including intermediate mesoderm cells [29, 30], renal progenitors [31], proximal tubule cells [32, 33], and podocytes [34]. Most of these studies have primarily relied on the expression of a set of markers and cellular morphology to validate the prospective renal cells generated via direct differentiation. While AQP1, AQP3, and apical microvilli were used to gauge proximal tubule epithelial cells differentiation, WT1, Synaptopodin, Podocin, and an arborized morphology were employed to evaluate PSC-derived podocytes. Typically, showing lineage-specific functionality is the most convincing evidence when it comes to the characterization of a lineage. One of the major challenges in renal cell differentiation is that the functionality of a specific renal cell type could hardly be recapitulated in the absence of an integral 3D tissue architecture. Alternatively, mouse embryonic kidney organotypic culture, either as an intact kidney rudiment or as a reassembled organoid, has been harnessed to study kidney development for several decades [35, 36]. The beauty of this culture platform is the feasibility to test the renal differentiation potential of any given cell ex vivo. In addition to standard marker analysis, these studies employed mouse embryonic kidney organotypic culture to evaluate whether human PSC-derived podocytes and proximal tubule epithelial cells can integrate into the mouse embryonic kidney. These analyses provided supportive, yet feeble, proof that these cells share some key properties with embryonic kidney cells. Furthermore, in the absence of systemic analyses, such as RNA-sequencing, it is still too early to conclude that we could consistently and convincingly generate renal lineages form human PSCs.

Meanwhile, protocols for differentiating human PSCs into neuronal lineages, cardiomyocytes, pancreatic, and hematopoietic lineages have been developed in a fast forward fashion, mostly attributed to the easily accessible

lineage-specific phenotypes. Kidney function is executed collectively by a large variety of cells that are organized with specialized structural conformation. Unlike the pancreas, where functional beta-cells alone are sufficient for reversing diabetic phenotypes [37], producing a single kidney cell type seems not adequate for regenerative medicine.

Kidney organoids

About one decade ago, the research teams led by Sasai and Clevers pioneered the organoid biology field by generating complex cortical [38, 39] and intestinal structures [40, 41] in a dish, respectively. In these studies, stem cells are exposed to minimum signals to garner lineage-specific information that allows them to self-organize into 3D miniature organs [23]. Kidney development entails the reciprocal interaction between two progenitor populations, metanephric mesenchyme (MM) and ureteric bud (UB) [42]. A growing understanding of the developmental trajectory of these two distinctive populations is shedding light on the possible means to recapitulate these processes in vitro [43, 44]. In addition, human PSCs expressing reporter genes under the control of kidney-specific gene promoters were successfully established via gene editing. Employing a GFP-OSR1 reporter human PSC line, Osafune and colleagues developed protocols for efficiently differentiating human PSCs into nephrogenic intermediate mesoderm cells that are capable of giving rise to multiple renal lineages, as well as integrating into mouse embryonic kidney organotypic culture [45, 46].

In 2013, the term "organoid" officially made its debut due to two turning point studies that generated brain organoid [47] and liver organ bud [48] from human PSCs. In the same year, direct differentiation approaches for steering human PSCs into MM [49] and UB [50] were separately developed by the Nishinakamura group and us. Both studies substantially improved the efficiency and accuracy of direct differentiation. More importantly, both human PSC-derived MM and UB established 3D embryonic kidney-like structures upon spinal cord co-culture or chimeric aggregate culture with mouse embryonic kidney cells, respectively [49, 50]. Moving forward, Little and colleagues developed a differentiation protocol that can generate both MM and UB derivatives in vitro [51]. Notably, these cells self-organized into 3D kidney epithelial tubule structures, in the absence of spinal cord or mouse embryonic kidney cells [51]. At the end of 2015, two landmark papers published by the Little group and Boventre group demonstrated for the first time that human PSCs can be efficiently differentiated into self-assembled 3D kidney organoids [52, 53]. These organoids, millimeters in diameter, are comprised of segmentally patterned nephron-like structures, stromal cells, and endothelial cells [52, 53]. Transcriptome analysis confirmed that the differentiation process is reminiscent of the developmental trajectory of the kidney, offering an in vitro model platform to investigate human kidney development [52, 54].

Missing pieces in the jigsaw puzzle and how to find them

The above studies open a new avenue of kidney organoid biology, ushering in many novel and intriguing studies in the last 5 years. One of the ongoing debates about kidney organoids is whether UB derivatives indeed exist. Most kidney organoid differentiation protocols do not strictly follow the developmental paradigm, in which MM and UB are developed separately. Typically, one single differentiation protocol gives rise to a repertoire of cells that self-organize into 3D nephron-like structures. To this point, the Nishinakamura team successfully differentiated mouse PSCs into UB progenitors that can construct high-order kidney organoids when assembled with mouse PSC-derived MM progenitors and mouse embryonic stromal cells [55]. This study not only brings forward a novel differentiation platform for generating 3D UB-like structures, but also highlights an unappreciated population for in vitro nephrogenesis, the stromal cells, which share a common intermediate mesoderm origin with MM [56]. During kidney morphogenesis, although cap mesenchyme and stromal cells maintain a strict lineage boundary [57], the cross talk between these two populations plays critical roles in promoting nephron progenitor differentiation via Fat4-mediated YAP nuclear export [58]. There are ongoing efforts in dissecting the heterogeneous cellular components in the kidney interstitium, as well as to understand the roles of insterstitium-derived signals in kidney development [59]. Unfortunately, there is still no report on direct differentiation of kidney stromal cells. It is likely that the stromal cells residing in the current kidney organoids may not represent the cognate stromal cells in the kidney in vivo [60].

As a filtration organ, the kidney cannot execute any function in the absence of a patent vascular network. Hence, another burgeoning question about the current generation of kidney organoids is how to make it vascularized. Unlike the liver bud, which is entirely vascularized by exogenous endothelial cells [48], kidney organoids contain some intrinsic endothelial cells [52], possibly because endothelial cells share the same mesoderm origin with kidney [61]. Various approaches have been developed to enhance kidney organoid vascularization. It is plausible that

the low abundance of endothelial cells is due to missing vascular endothelial growth factors (VEGFA) provided by neighboring cells within kidney organoids. While the Freedman group directly injected VEGFA into the kidney organoid culture media to enhance vascularization [62], we chose to coordinate the intraorganoid signaling communication between podocytes and endothelial cells to fine tune the abundance of vascular endothelial cells [63]. However, within a typical static kidney organoid culture, the intrinsic vasculature has a low chance to invade into the glomerular corpuscle to establish an integral glomerular filtration barrier, which constitutes the key functional element of the kidney [62, 63]. Within blood vessels, blood flow-accompanied sheer stress is a critical determinant for the sculpting of capillaries [61]. To address this issue, most research teams implanted kidney organoids [64, 65] or nephron progenitors [66] beneath the renal capsule or subcutaneously. Kidney organoids anastomosed with the host circulation system shortly after implantation [63, 64, 66]. Histology analyses demonstrated that an existing circulation system enables the formation and perfusion of glomerular capillary tufts [63–66]. In some studies, vascular endothelial cells residing in the implant were originated from the host instead of the organoid itself [64], probably because of the low abundance of organoid intrinsic vascular endothelial cells. The resident vasculature within our kidney organoids successfully invaded into the glomeruli to establish the filtration barrier [63]. Alternately, the Morizane group and Lewis group developed a microfluidic device for kidney organoid culture, which can foster glomerular vascularization in vitro, representing a milestone toward in vitro kidney organoid functionalization [67].

Over the last 5 years, rapid development of single-cell analysis tools has enabled us to scrutinize the cellular composition of current kidney organoids, and to establish bench marks against in vivo kidneys at single-cell resolution. Not unexpectedly, most human PSC-derived kidney organoids resemble the first-second trimester developing human kidney [52, 63, 68]. Although implantation into a host can help promote maturation, it cannot push the kidney organoid into subsequent developmental stages [63]. Despite the existence of segmentally patterned nephron-like structures, the expression of functional transporters and receptors is far from being satisfactory in kidney organoids. For example, SLC5A12 and UMOD expression is almost undetectable in different versions of kidney organoids [60]. Due to the absence of a co-differentiating immune system, tissue-resident macrophages are largely missing, despite their critical role in shaping the developing kidney [69]. In addition to the above-mentioned defects, human PSC-derived kidney organoids contain a large number of unwanted "weeds," such as neuronal cells, muscles, and many uncharacterized mesenchymal cells [60, 62, 70]. A systemic approach for eliminating unwanted cells is currently missing, though various methods are being developed to reduce off-target cells [60, 70].

The many caveats existing within the kidney organoids are similarly observed in other types of organoids derived from human PSCs (Fig. 27.2), indicating a common knowledge gap in our understanding of organ development at late gestation and early postnatal stages. Ongoing global efforts in mapping the transcriptome, genome, and epigenome of developing, maturing, homeostatic and pathological human organs at single-cell resolution will undoubtedly unveil the knowledge that was previously unknown due to a lack of tools. Furthermore, the complexity of the tissue microenvironment is gradually unveiled, as well as its crucial role in organ development, homeostasis, and disease pathogenesis. In particular, physical features of the microenvironment are receiving increasing attention, due to their influence in cell fate determination [68]. The availability of synthetic hydrogels and microfluidics provide unprecedented opportunity for sculpting the physical environment for optimal in vitro organogenesis.

Disease modeling using kidney organoids—Playing to its strength

Historically, mouse models are most heavily used for studying human diseases. Compared with other animal models, such as zebrafish and *Drosophila*, mouse is considerably closer to human from an evolutionary standpoint. Thus the mouse model is often accredited for its faithful recapitulation of human phenotypes. Most of our understanding of kidney development is accumulated from studies using genetic mouse models. Although mouse and human share many common features, as far as kidney development, structure, and function are concerned, studies from recent years have unveiled increasing divergence between mouse and human kidney development and disease. For example, mice have a birth nephron number of ~ 130,000 per kidney, while humans are born with approximately 1 million nephrons per kidney [42]. The McMahon group recently demonstrated a divergence in key nephron progenitor factors SIX1 and SIX2 regulation between mouse and human [71]. Taking polycystic kidney disease (PKD) as another example, despite the great number of available animal models of PKD, there is none that closely resembles human PKD presentation, in terms of severity and culminating time window [72]. Specifically, while $PKD1^{-/-}$ mice develop severe renal cysts during the embryonic stage, $PKD1^{+/-}$ mice in many instances merely develop microscopic renal cysts in the adult [72]. Furthermore, the genetic complexity of PKD makes it impossible to generate matching mouse models for every pathogenic mutant identified in patients [73]. Therefore, an alternative model platform is required for recapitulating human kidney diseases.

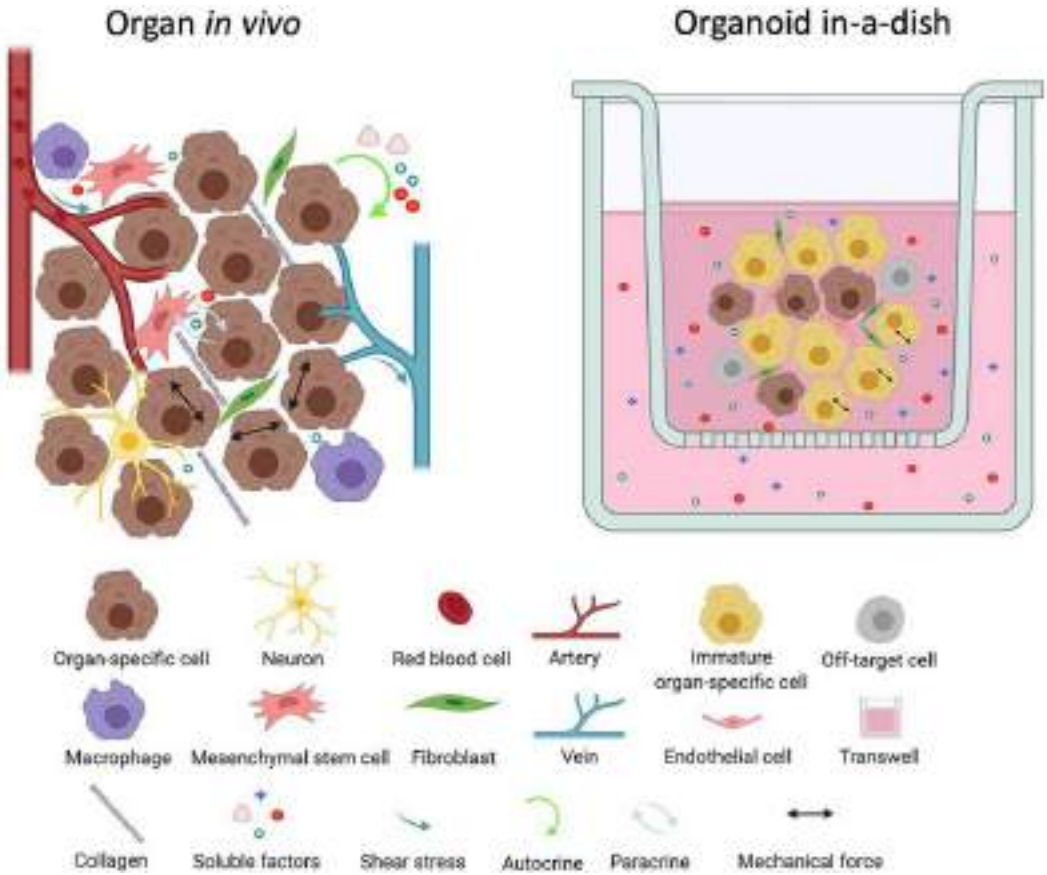

FIG. 27.2 Comparison between organoid in a dish with in vivo cognate organ.

Though using human PSC-derived kidney organoids for regenerative medicine may not be as feasible as we would like, we should not ignore the power of this model, in particular, with regards to its relevance to human renal biology and its amenability for genetic manipulation. Before we set out to establish a disease model, we should first question its feasibility. Most kidney diseases manifest in the adult, and not at a fetal stage. Single-cell transcriptomic analysis, alongside genome-wide association study (GWAS) revealed that kidney organoids are missing many genes associated with kidney diseases [60, 74]. In addition, some kidney disease phenotypes, such as extracellular matrix deposition during fibrosis, might be confounded with in vitro culture defects. Furthermore, variability among organoids might make it even more difficult to standardize and quantify the observed disease phenotypes.

Among various types of kidney diseases, PKD has been most successfully emulated using human PSC-derived kidney organoids, thanks to its easily definable cystic phenotype. Due to the variable differentiation outcome observed in different patient-derived iPSC lines, the Freedman group generated PKD1$^{-/-}$ and PKD2$^{-/-}$ human PSC lines to study cytogenesis [75–77]. Employing an adherent-free culture approach, PKD1$^{-/-}$ and PKD2$^{-/-}$ PSCs rapidly developed huge tubule cysts [75]. Using this in vitro cystic organoid model, they embarked on identifying novel pathways underlying renal cyst formation [62]. However, in the real world, renal cysts develop within a solid organ instead of in liquid. Based on our kidney organoid differentiation platform, we employed a stress paradigm to develop a cystic kidney organoid model that can specifically recapitulate tubule cyst formation in PKD patient iPSC-derived kidney organoids, instead of the isogenic gene-corrected organoids [63]. Renal cysts can originate from different nephron segments. Freedman's and our cystic kidney organoid models primarily emulate proximal tubule originated cysts [63, 75]. However, in postnatal autosomal recessive PKD (ARPKD) and some autosomal dominant polycystic PKD (ADPKD) patients, cysts originate from UB/collecting ducts. To address this, the Nishinakamura group has likewise employed a stress paradigm to develop a cystic UB organoid model that can mimic cyst formation in both gene-edited (PKD1$^{-/-}$) and ADPKD patient iPSC-derived UB organoids [78]. The availability of a segment-specific cystic organoid model provides a new avenue for us to dissect the mechanism behind segment-specific cyst formation, as well as to accurately test the efficacy of candidate drugs for cyst inhibition. Further studies are required to investigate additional phenotypes that are typically associated with PKD, such as defective energy

metabolism and ciliary defects. To fulfill prospective drug screening, it will be necessary and practical to translate qualitative phenotype assessment into quantitative phenotype measurement.

In vitro nephrogenesis II—Capturing kidney progenitors using a synthetic niche

On the one hand, using human PSC-derived kidney organoids for organ regeneration remains a far-fetched goal. On the other hand, a postnatal kidney stem cell population that can give rise to an entire nephron has not been identified in human or mouse. Such a multipotent kidney progenitor population only exists during kidney embryogenesis [28]. In mouse, nephron progenitors are exhausted in the first few days after birth, while in human, this population is depleted by 34–36 weeks of gestation [79]. Is it possible to capture and amplify this precious stem cell population in vitro for long term, similar to what scientists have been trying to do with hematopoietic stem cells? Within the in vivo niche, maintenance of nephron progenitors requires the signaling input from their neighbors, including UB and stroma, as well as from themselves in an autocrine manner [80]. A delicate balance of multiple signaling pathways, including BMP, FGF, and WNT, is needed to maintain the nephron progenitor pool during nephrogenesis [81–83]. One study by the Little group demonstrated that nephrospheres could be successfully derived from mouse embryonic kidneys but not adult kidneys using defined medium, though they quickly lost their epithelization capacity [84].

Oxburgh and colleagues developed a synthetic niche for culturing mouse nephron progenitors of different developmental stages for a considerable period without losing the capability to self-renew and differentiate [85]. The same medium can also culture and amplify human ESC-derived nephron progenitors for a brief period [85]. To better mimic the in vivo tissue microenvironment, we developed a 3D synthetic niche for infinitely culturing mouse nephron progenitors without compromising self-renewal and nephrogenesis [86]. More importantly, our 3D nephron progenitor culture platform is amenable to genetic manipulation [86]. Due to the divergence between human and mouse nephrogenesis, we modified the medium recipe to accommodate human nephron progenitors derived from second trimester human embryos, as well as human PSC-derived nephron progenitors [86]. This culture approach has substantially broadened our ability to handle nephron progenitors from different sources. Although these native nephron progenitors display a far superior capacity in nephrogenesis both in vitro and in vivo, there is a lack of evidence that their derivatives could surpass the developmental barrier encountered by human PSC-derived kidney organoids. Future studies are needed to address this critical issue before we could regard this approach as a feasible alternative for in vitro kidney regeneration.

Meanwhile, we should not forget the other progenitor population that gives rise to the collecting duct system, ureteric bud (UB). There has been a general belief that successful in vitro nephrogenesis requires the assembly of both nephron progenitors and UB. Yanagawa and colleagues successfully propagated single UB cells in vitro that can resume branching morphogenesis [87]. These in vitro-expanded UB cells are capable of inducing MM condensation and epithelization [87]. However, this culture platform was not able to maintain UB cell culture for long term. Ongoing studies by Li and colleagues have formulated a defined medium that can beautifully maintain both mouse and human embryonic kidney-derived UB cells in 3D in vitro culture for long term (https://www.biorxiv.org/content/10.1101/2020.04.27.049031v1). These UB cells, amenable to genetic manipulation, remain competent in branching morphogenesis and inducing MM to undergo mesenchyme to epithelial transition (MET) and further differentiate into nephron-like structures. Although more needs to be done to perfect these culture platforms to better accommodate human PSC-derived MM and UB cells, these synthetic tissue microenvironments undoubtedly offer an alternative means to consider in vitro nephrogenesis for regenerative medicine.

In situ kidney cellular regeneration

In a mature organism, cell identity remains stable throughout its lifespan. Most vital organs, such as the pancreas, brain, and kidney do not possess the capability for postnatal neoorganogenesis (organ regeneration). To compensate for this limitation, most mammalian organs employ cellular regeneration to restore the lost or damaged cellular components. During the last few years, it has become increasingly clear that whereas cell fate conversion during homeostasis follows a prescribed developmental path, injury usually makes cell fate substantially more plastic. In injured organs, cell fate plasticity typically adopts two different formats of strategies, dedifferentiation, and transdifferentiation. Due to the fact that transdifferentiation has not been explicitly demonstrated as a regenerative strategy in the mammalian kidney, we will focus on dedifferentiation here.

Although mammalian nephron progenitors are depleted during the perinatal stage, abolishing neonephrogenesis in postnatal mammals, the adult kidney possess a remarkable capability to restore the damaged nephron epithelium [1]. In the last decade, there has been a collective effort in deciphering the mechanisms underlying adult kidney regeneration, which may lead to the identification of new therapeutic targets and strategies. Acute kidney injury, a highly prevalent medical condition, can lead to the future development of chronic kidney disease if the kidney regeneration process failed to replenish denuded tubules [88]. Though there is a long-standing question as to whether a resident tubular progenitor population exists or not, we are quite sure that intrinsic tubular cells, instead of an extrarenal cell source, restore the structural integrity of the damaged tubules. In 2008, Humphreys and colleagues generated Six2-GFPCre$^+$; Z/Red$^+$ mice to track cap mesenchyme progenies during kidney regeneration upon acute kidney injury [89]. This landmark study demonstrated that injured tubules were repaired exclusively by the proliferation of Six2 descendants [89]. On the other hand, identification of label-retaining cells (LRCs) in kidney papilla [90, 91] and collecting duct [92] has led to the long-standing belief that these cells may act as the resident stem cell population to regenerate damaged tubular epithelium, due to the observation that LRCs proliferate and contribute to embryonic [93] and neonatal mouse kidneys [92], as well as postinjury kidneys [90]. An elegant pulse-chase assay argued that papillary LRCs do not selectively proliferate or migrate after ischemic reperfusion injury, despite the substantial increase in cortex and medullary tubular proliferation [94]. Nonetheless, a recent study showed that Zfyve27$^+$ renal papillary LRCs are activated to regenerate the damaged nephron epithelium only after severe kidney injury [95]. Notably, there is a lack of evidence that a designated intratubular progenitor population is responsible for tubule regeneration [94]. Post injury, surviving epithelial cells lose characteristic tubular features, such as the basolateral distribution of Na/K/ATPase, while re-express Pax2, a critical regulator for kidney development supporting a dedifferentiation phenotype [94]. Different from many other organs, in that, dedifferentiation leads to the generation of multipotent progenitors, kidney epithelium development [96] and regeneration [97] always takes place within segment-specific boundaries.

An alternative model of kidney cellular regeneration is established based on the existence of an intratubular progenitor population. These progenitors, a subset of parietal epithelial cells (PECs) expressing CD133 and CD24, were first identified in the urinary pole of the Bowman capsule in human kidneys [98]. This stem cell population possesses prominent self-renewal and multilineage potency in vitro and can protect against acute renal failure in mice by giving rise to tubule epithelial cells [98]. Follow up studies further elaborated that CD133$^+$ CD24$^+$ CD106$^+$ renal progenitors located at the urinary pole of the Bowman capsule can give rise to both podocytes and tubular epithelial cells [99], while CD133$^+$ CD24$^+$ CD106$^-$ renal progenitors located in the proximal and distal tubules proliferate upon tubular injury to specifically restore damaged tubular epithelial cells [100]. Of note, although these human kidney progenitor cells can rescue mouse kidney injury, equivalent populations have not been identified in mouse [1]. Interestingly, one study identified CD24$^+$ CD133$^+$ Vimentin$^+$ cells in the resting state human kidney; whereas CD24$^+$ cells were absent in healthy rat kidneys [101]. Post injury, the human kidney displayed an increased proportion of CD24$^+$ cells, while the rat kidney established a de novo CD24$^+$ population, which presumably may contribute to tubular regeneration [101]. Co-expression of Vimentin and Kim1 in CD24$^+$ CD133$^+$ cells suggested that these cells may not represent a putative stem cell population, but rather be a consequence of dedifferentiation.

This debate continues as we accumulate new evidence and knowledge. In a study using proximal tubule-specific SLC34a1-GFPCreER^{T2} transgenic mice for tracing post injury lineage tracing, no dedicated kidney stem cell population was identified, whereas dedifferentiation and acquisition of stem cell marker expression, such as CD24, CD133, and Vimentin were observed in injured tubular epithelial cells [102]. Meanwhile, another study employed PEC-rtTA transgenic mice to label the scattered tubular cell population [103]. This lineage tracing study came to a similar conclusion that these scattered tubular cells do not represent a dedicated progenitor population, but rather assumed a dedifferentiated phenotype that can be adopted by almost any proximal tubular cell upon injury [103]. Nonetheless, new knowledge continually revises our existing interpretation of kidney cellular regeneration. The recent identification of a Sox9$^+$ population within the postinjury kidney highlighted the possibility that a subpopulation of tubular epithelial cells may robustly regenerate the damaged tubular epithelium alongside the well-established dedifferentiation mechanism [104, 105].

The existence of adult kidney stem cells has long been debated, though studies based on different hypotheses converge on many similar aspects (Fig. 27.3). Based on both theories, the cells in charge of tubular regeneration present characteristic stem cell marker expression and diminished epithelial markers. Notably, while Pax2 re-expression was observed in damaged proximal tubules that are undergoing dedifferentiation, Pax2 expression also marks CD133$^+$ kidney progenitors that specifically give rise to podocytes during postnatal kidney growth and regeneration [106]. A recent study identified Pax2$^+$ progenitor cells that scattered across multiple tubular segments [107]. These observations strongly indicate that the two seemingly disparate models may not necessarily be mutually exclusive.

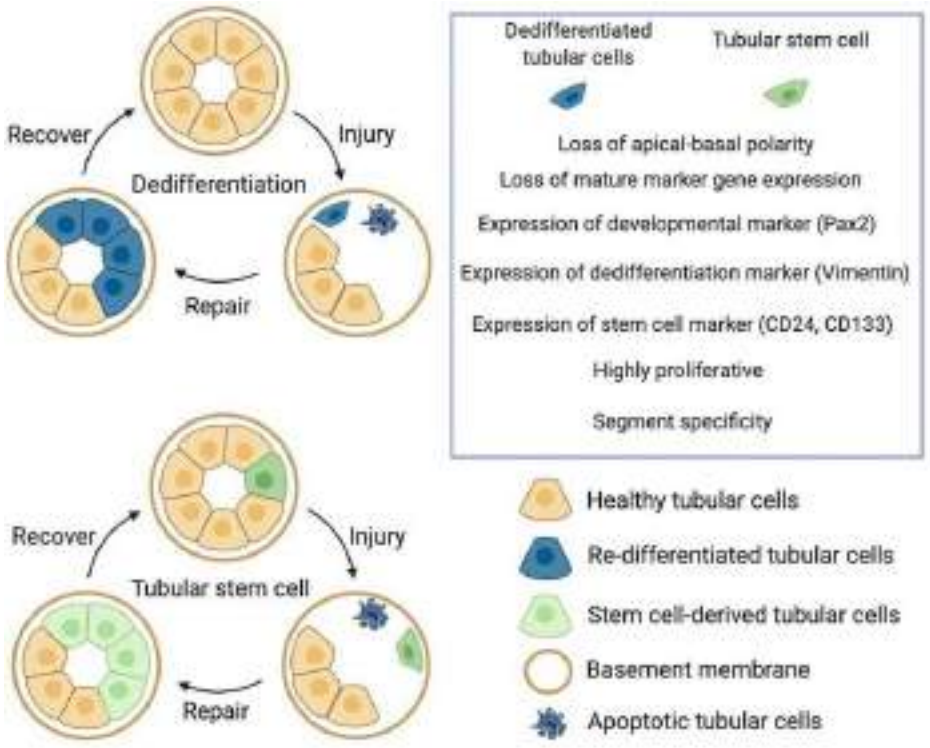

FIG. 27.3 Divergence and convergence of two theories for adult kidney cellular regeneration.

Taking into consideration the recently identified Sox9^{+} tubular subpopulation, kidney regeneration may be a highly heterogeneous process that involves multiple pathways working in concert on a case-by-case basis. The emergence of novel lineage tracing tools, alongside single-cell transcriptomic analysis and ATACseq (Assay for Transposase-Accessible Chromatin using sequencing), will help us to dissect the detailed mechanism of cellular regeneration in the mammalian kidney. With the current body of knowledge about the putative cell populations that are responsible for adult kidney regeneration, researchers are working toward unveiling targetable pathways for enhancing kidney cellular regeneration [106, 108, 109].

In vivo lineage reprogramming

Exploiting cell fate plasticity in vivo

Strictly speaking, in vivo lineage reprogramming is not an entirely new concept. iPSC reprogramming has rekindled an interest in early studies showing that transcription factors can directly reprogram cells from one lineage to another [110]. Based on these early studies and recent observations, such as in vivo alpha-beta cell conversion, biologists have fostered the concept of regenerative in vivo lineage reprogramming, which aims to convert tissue resident cells into the cell types that are lost due to disease or injury. Unfortunately, in vivo lineage reprogramming rarely takes place naturally in mammals. The current definition of in vivo lineage programming primarily refers to forced cell fate conversion for organ regeneration.

There are a few milestones that define successful in vivo lineage reprogramming. The first important decision to make is the choice of source cell type that will be reprogrammed. Practically, geographic location and intrinsic function of source cells, as well as their quantity, all need to be considered. There is extensive evidence supporting the choice of a source cell that is both developmentally and geographically close to the target cell type, such as acinar or ductal cells for beta-cells and astrocytes for neurons [111]. There has been much enthusiasm in reprogramming postinjury scar-forming cells into functional tissue-specific cells. In two recent studies, hepatic myofibroblasts and wound-resident mesenchyme cells were successfully reprogrammed into hepatocytes [112] and keratinocytes [113] to attenuate pathogenic phenotypes in mouse.

The second milestone, which is to define a reprogramming route, usually needs to be deliberated once the source cell type is selected. This involves choosing the optimal minimum set of reprogramming factors that are expected to override the existing cell identity of the source cell and allow them to assume the desired cell fate. These reprogramming factors are mostly transcription factors and microRNAs that play determinant roles in defining the identity of target cells during development [111, 114]. It is of paramount importance to select an efficient and safe approach to deliver the reprogramming factors in vivo. Currently, adenovirus and adeno-associated virus are promising candidate carriers of reprogramming factors, though there may be some difficulties in realizing tissue-specific delivery [115]. In several interesting studies, lineage reprogramming was achieved by employing small molecules [116–118], highlighting a promising alternative with higher clinical feasibility.

Some lineage conversion routes involve an intermediate stage allowing for amplification of the desired cell type, while most occur by direct conversion from source cell to the desired cell type. Typically, lineage reprogramming requires a set of pioneering factors to reorganize the epigenome of source cells, initiating the adoption of a different cell identity [119]. During iPSC reprogramming, Yamanaka factors substantially rewire the epigenome of the parental cells, leading to an intermediate plastic cell state before reaching pluripotency [120]. One branch of lineage reprogramming studies attempted to take a "shortcut" via coupling partial iPSC reprogramming with growth factors/chemicals-guided differentiation [121]. Although there are ongoing debates on the feasibility of this approach [122, 123], there are studies demonstrating successful lineage conversion employing partial reprogramming [124].

Some additional factors to consider include phenotypic resemblance between the reprogrammed cells and desired cell types, as well as efficient integration of reprogrammed cells into the injured organ. Unfortunately, none of the available in vivo reprogramming approaches is anywhere close to a clinical trial, even though there has been intensive research over the last decade. However, it does not take away the potential of this approach as an alternative to cell replacement and gene therapies.

Is in vivo lineage reprogramming a far-fetched goal for kidney regeneration?

In vivo reprogramming has been most extensively investigated in the pancreas, central nervous system, liver, and heart, possibly because one specific cell type may be able to compensate for a devastating injury-induced phenotype. When it comes to the kidney, it is an unfortunate fact that so little has been achieved to date. One possible reason is that the kidney contains approximately 30 different types of cells organized into a complex 3D structure. Furthermore, kidney function is heavily dependent on its structural integrity. Hence, researchers refrain from conjecturing that generating one kidney cell type via in vivo lineage reprogramming could suffice for kidney regeneration. It is commonly acknowledged that podocytes and proximal tubular epithelial cells are the most vulnerable cell types in the kidney. Due to their geographic location, it may be more practical to manipulate the intrinsic cellular regeneration capability, rather than try to convert another cell type in the vicinity. Nonetheless, one recent study successfully transdifferentiated human fibroblasts into kidney tubular epithelial cells that resemble human proximal tubular cells in terms of morphology and functionality in vitro [125]. However, there is a lack of in vivo analysis showing that these reprogrammed kidney tubular epithelial cells can restore kidney structure and function. More recently, it has been shown that Renin$^+$ cells can transdifferentiate into podocytes after massive podocyte injury [126], suggesting for the first time that in vivo lineage reprogramming may be an intrinsic feature of some renal lineages.

Another kidney cell population of great regenerative potential is nephron progenitors, which can give rise to the entire nephron from podocytes to connecting tubules. Unfortunately, this multipotent nephron progenitor population does not exist in the postnatal mammalian kidney. The Little group established a transdifferentiation approach to convert HK2 cells (human proximal tubular epithelial cells) into nephron progenitor-like cells that can contribute to the cap mesenchyme in chimeric aggregate culture with mouse embryonic kidney cells [127]. Chronic kidney disease presents the gradual decline of nephron numbers and accumulation of fibrotic tissue. It would be really appealing if fibrotic tissue could be reprogrammed into nephron progenitor-like cells for neonephrogenesis. Much remains be done to realize efficient lineage reprogramming toward expandable nephron progenitor-like cells. We also need to keep in mind that the adult kidney no longer possesses a proper niche to steer nephron progenitor-like cells into functionally mature nephrons, and to connect the newly formed nephrons to the collecting system. It remains an open question whether an in vivo lineage reprogramming approach is suitable for neonephrogenesis in the adult mammalian kidney, or should it be twisted toward boosting kidney cellular regeneration via epigenetic reprogramming [128].

Interspecies chimeras for kidney regeneration—A legendary mission?

Interspecies chimeric assays used to be employed for investigating the developmental potential of stem cells [129–132]. In 2010, a landmark study by Nakauchi and colleagues demonstrated for the first time that a rat pancreas can be generated in mice by injecting wild-type rat ESCs into $Pdx1^{-/-}$ mouse blastocysts [133]. This study introduced the concept of regenerative chimeric complementation, which aims to generate an organ by injecting one animal's PSCs into another animal's embryo that has been genetically modified to vacate the developmental niche of this organ. In comparison with traditional chimeric assays that are highly random, chimeric complementation represents a strategy that can selectively enrich chimerism in a specific organ or tissue. In the last decade, interspecies chimeras have been regaining popularity in regenerative medicine, with the hope of generating transplantable human organs within large animals [134, 135]. The advance of CRISPR-Cas9 gene editing tools makes it highly feasible to remove gene(s) that are required for the development of a specific organ. Meanwhile, the appreciation of dynamic pluripotency has triggered scientists to establish conditions for capturing and maintaining a spatiotemporally defined pluripotent state in vitro [135]. Nonetheless, it remains technically challenging and ethically debatable to pursue organ generation using chimeric complementation [132, 136].

So far, successful interspecies chimeric complementation has only been achieved between closely related species, including goat-sheep [137], rat-mouse [133], and cow-zebu [138], suggesting that a short evolutionary distance between the donor stem cells and host embryo is desirable. In addition, a matching developmental timing between the donor stem cells and host embryo is essential for efficient chimerism [139]. Postimplantation mouse epiblast-like stem cells (EpiSCs) are not able to efficiently integrate into the preimplantation host blastocyst inner cell mass (ICM) [140], whereas they can robustly graft into postimplantation mouse epiblast [141]. Collectively considering all these major obstacles, we generated the first human-pig chimera by injecting naïve or intermediate human PSCs into pig blastocysts, albeit with low efficiency [136].

Due to the technical challenges and ethical considerations, most chimeric complementation studies are conducted in rodents to obtain a proof-of-concept. In addition to the two primary factors, evolutionary distance and developmental synchrony, which determine the efficiency of chimerism, another obstacle to overcome is how to vacate the developmental niche of an organ in the host embryo. To this point, the pancreas is a relatively convenient organ, because its development is predominantly dependent on only one gene, Pdx1 [133, 142]. Unfortunately, such convenience is not very often seen in other organs. In a recent study, wild-type donor mouse PSCs successfully complemented the vacant lung-related niche in host mouse embryos. Although this strategy successfully rescued the otherwise devastating lung deficiency in $Ctnnb1^{cnull}$ and $Fgfr2^{cnull}$ mutant mice, the current strategy is not sufficient to produce a functional lung entirely formed by donor cells [143].

Kidney development involves a complex network of regulators, making it difficult for us to select the principle driver gene(s), the absence of which will eliminate the entire kidney. A $Sall1^{-/-}$ mouse blastocyst was developed to accommodate wild-type mouse PSCs [144], which contributed to all kidney lineages (all epithelial derivatives of MM and most stroma) with high efficiency, except for structures that are not under the direct regulation of Sall1, such as the collecting duct [144]. Since Sall1 also plays critical roles in neuronal development, chimeric complementation does not always rescue mice from embryonic lethality, possibly due to the variable chimerism proportion [144]. Recently, this strategy has been extended from intraspecies (mouse-mouse) to interspecies (mouse-rat) chimeric complementation. Although rat PSCs failed to give rise to kidneys in anephric $Sall1^{-/-}$ mice, mouse PSCs can efficiently differentiate into metanephric mesenchyme in rats, followed by specification into other MM derivatives [145]. Different from the earlier study, mouse ESCs contributed to the collecting duct and renal vasculature as well, albeit with lower efficiency, because $Sall1^{-/-}$ rats remain competent in generating these cells.

We should also consider several other caveats of the current chimeric complementation approach, which have not been extensively investigated. Organs generated by chimeric complementation still contain generic cell types derived from the host, such as vasculature, neurons, and probably stroma. These generic cells will trigger an immune response after the organ is transplanted into a recipient. Hence, ablation of both organ-specific cells and generic cell types may be necessary for generating an organ entirely formed by donor PSCs. On the other hand, when an organ of one species is growing within the body of another species, prospective immune rejection is expected, despite the physiological need of this organ. These unresolved obstacles will need to be addressed before we could move forward with chimeric complementation-based organ generation. Although it is far from being optimal, this unique strategy may offer an unexpected alternative approach for generating transplantable kidneys in the future.

Conclusion

The last 10 exciting years have borne witness to many advances in our understanding of cell fate plasticity, urging us to revisit an organ's capability to regenerate. Meanwhile, the development of novel experimental tools has substantially expanded our capability to investigate cell fate plasticity, as well as to manipulate cell identity for organ regeneration. Although the mammalian kidney is defined as a nonregenerating organ, it is remarkably potent in restoring a damaged epithelial population via intrinsic cellular regeneration. A thorough understanding of this regenerative response is leading to the development of therapeutic approaches in promoting kidney regeneration. Although human pluripotent stem cell-derived renal cells/organoids are not yet optimal for kidney regeneration, they offer a powerful means to recapitulate kidney diseases in the presence of a 3D tissue microenvironment in vitro. The availability of other seemingly far-fetched approaches like in vivo reprogramming and chimeric complementation opens new avenues that may 1 day lead to unprecedented methods for realizing kidney regeneration.

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CHAPTER

28

Therapeutic cell reconditioning

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Definition of cellular reconditioning

The well-established concept of cell reprogramming is being supplemented by the emergence of a novel concept of cell reconditioning. In the past, the term "reconditioning" was occasionally used in relation to sport medicine, space flights, but more recently, in epigenetic reconditioning of tumor cells and in transplantation medicine to improve functions of suboptimal grafts of lung, kidney, or liver by ex vivo perfusion using pharmaceuticals. These include treatments with inhibitors of peroxynitrite and poly(ADP-ribose)polymerase [1] or ex vivo venous oxygen persuflation with nitric oxide [2–5]. In the current iteration, however, I use the term "cellular reconditioning" as not only incorporating the above cases but mainly to stress the point that *refurbishment of some pathogenically pivotal intra- or extra-cellular elements, damaged in the course of disease and impeding restoration* represents a viable option for promoting regeneration. Defaulting cellular organelles or aberrant extracellular environment may be detrimental to organ functioning; with their restoration cells and organs become *reconditioned* toward a more complete regeneration after distress. Coincidentally, in many instances cell reconditioning leads to its reprogramming, as is the case with epigenetic reconditioning of tumor cells subjected to demethylating agent 5-azacytidine promoting their differentiation to somatic lineage [4]. The proposed principal pathways for cell reconditioning are schematically illustrated in Fig. 28.1.

Cellular structures and metabolic pathways hampering regeneration

To address the pathways for reconditioning, it is necessary to first recognize the mechanisms that participate in resistance to regeneration. This group of factors includes some defaulting cellular organelles, metabolic perturbations, or aberrant extracellular environment, as detailed below.

- *Mitochondriopathy*, a frequent companion of diverse renal diseases, results in mitochondrial cytopathy. The latter can be caused by inherited or acquired mitochondriopathy, as reviewed in detail elsewhere [6]. Inherited cases may be due to mutations in mitochondrial or nuclear DNA and lead to clinical manifestations of focal-segmental glomerulosclerosis, MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes), Fanconi and Bartter-like syndrome with or without myoclonic epilepsy, and rugged muscle fibers, as well as Pearson's, Kearns-Sayre, and Leigh syndromes. Some tubulopathies have a tendency to form cysts. In several models of mitochondrial dysfunction, such as electron transport chain inhibitors or depletion of the mitochondrial chaperone HSPA9, Wiley et al. [7] demonstrated decreased NAD^+/NADH ratio and development of premature senescence, the so-called mitochondrial dysfunction-associated senescence—MiDAS. One of the conditions associated with the loss of mitochondrial-encoded subunits of oxidative phosphorylation, which is induced by the decline in the nuclear availability of NAD^+ and leads to the accumulation of HIF-1α under normoxic conditions (the so-called a pseudohypoxic state), is characteristic of aging [8]. Acute kidney injury and chronic kidney disease belong to a large group of acquired mitochondriopathies. There is strong evidence that mitochondriopathy is an impediment to kidney regeneration (reviewed in Ref. [9]).

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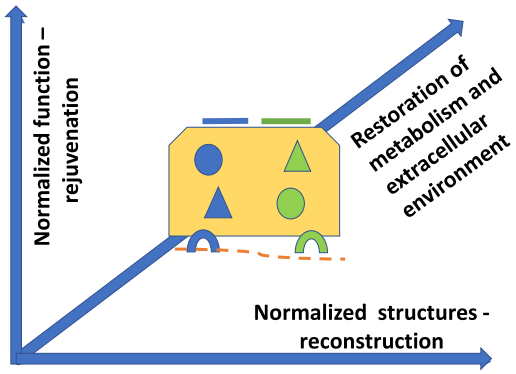
Normalized function – rejuvenation
Restoration of metabolism and extracellular environment
Normalized structures - reconstruction

FIG. 28.1 Modes of cell reconditioning. The cross-point equates to cell demise. Distinct cell organelles are depicted as different geometrical forms—defaulting ones in *blue* and restored ones in *green*.

- *Lysosomal and peroxisomal dysfunction*—These phenomena are intricately associated with diverse kidney disease [10–13]. We proposed [14] that "frustrated autophagy" due to the stress-induced collapse of pH gradient results in the accumulation of defective peroxisomes and mitochondria (a result of impaired pexophagy and mitophagy, respectively) leading in turn to tissue impregnation with very long-chain fatty acids escaping β-oxidation and defective oxidative phosphorylation.
- *Defective glycocalyx*—Glycocalyx of endothelial cells is an early victim of ischemia-reperfusion, endotoxemia, some infectious diseases; it is gradually degraded in commonly encountered cardiovascular and metabolic diseases; and it is accompanied by global endothelial dysfunction (reviewed in Ref. [15]). We proposed the concept of a vicious circle type relationship between endothelial dysfunction and the loss of glycocalyx. This concept has been formulated to explain the tight association between the two conditions and to emphasize the need for therapeutic interventions targeting both (see below), as summarized by Zhang et al. [15]. The stigmata of endothelial cell dysfunction, such as reduced NO bioavailability, enhanced production of reactive oxygen species, activation of sheddases, impaired exocytosis of lysosome-related organelles—all trigger degradation of glycocalyx, which in turn leads to defective shear stress-induced activation of eNOS and further decline in NO bioavailability, shedding of components of glycocalyx endowed with pro-inflammatory properties, loss of glycocalyx-bound growth and survival factors, thus fueling the vicious circle.
- *Metabolic pathways:*
  - *Acetyl-CoA deficiency and histone acetylation*—Acetyl-CoA availability regulates histone acetylation, whereas sirtuin 1 deacetylation activity counterbalances it. Histone acetylation and open chromatin structure are increased by aerobic glycolysis, whereas acetyl-CoA is consumed in the Krebs cycle, thus resulting in reduced availability of the 2-carbon acetyl and thus leading to the net chromatin deacetylation. Under pathological conditions, both are dysregulated, potentially affecting regenerative processes.
  - *Impaired nicotinamide adenine dinucleotide (NAD^+) generation*—This coenzyme in redox reactions and a cofactor of sirtuins, ADP-ribose transferases, and poly(ADP-ribose) polymerases, and cyclic ADP-ribose synthases is synthesized from nicotinic acid mononucleotide via Press-Handler pathway and from tryptophan via the de novo kynurenine pathway (reviewed in Ref. [16]). It can be also recycled in a salvage pathway from nicotinamide. NAD^+ participates in nearly one-fourth of all biochemical reactions listed in Kyoto Encyclopedia of Genes and Genomes [17]. Conditions associated with increased DNA damage, oxidative stress, aging [all inducing poly(ADP-ribose) polymerases], as well as inflammatory diseases [all reducing nicotinamide phosphoribosyltransferase] result in the reduced NAD^+ biosynthesis, enhanced competitive consumption by poly(ADP-ribose) polymerases, and eventuating in NAD^+ deficiency. The latter results in the reduction of already repressed sirtuins activity and further metabolic deterioration that limits regenerative processes.
  - *Microenvironmental impediments to regeneration*—Ambient environmental cues, such as cell secretomes, senescence-associated secretory products (SASP), cyto- and chemokines, and extracellular matrix proteins have a profound effect on regeneration and may occasionally represent therapeutic targets. An example of reconditioning of cell environment to achieve reprogramming is provided by the finding that in vivo blockade of CXCL12 in mice with Adriamycin-induced nephropathy salvages renal function by inhibiting Notch signaling in podocyte progenitors [18]. SASP affect the phenotype of cells of origin, as well as neighboring cells. The components of SASP are profibrotic and proinflammatory agents, such as interleukins 1, 6 and 8, connective tissue growth factor, transforming growth factors, plasminogen activator inhibitor 1, MCP-1, GM-CSF,

G-CSF, HGF, IGF, PDGF, VEGF, MMPs (reviewed in Refs. [19, 20]) acting in paracrine, autocrine, and juxtacrine fashion. Matsumoto et al. [21] examined secretomic messages promoting the loss of endothelial cell phenotype and acquisition of mesenchymal phenotype. We attempted to reverse endothelial-mesenchymal transition in the course of fibrotic kidney diseases, like unilateral ureteral obstruction or chronic phase of folic-acid-induced nephropathy. Exposure of TGF-beta-stimulated fibroblasts to the endothelial progenitor cell-free extract halted their conversion to myofibroblasts; similarly, fibrogenesis was attenuated in the above fibrotic models by renal subcapsular injection of this extract. One of the components of the extract was identified as leukemia inhibitory factor (LIF), and we found that it is capable of preventing the development of contractile phenotype of activated fibroblasts. It has also induced some pluripotency factors—Nanog and c-Myc, indicative of a partial pluripotency intermediate state. In vivo, a powerful, LIF receptor-independent agonist of gp130/STAT3 pathway, hyper-IL-6, prevented renal fibrosis.

In dysfunctional senescent endothelial cells, one of the detectable messages is represented by collagen XVIII and its C-terminal antiangiogenic fragment, endostatin [22]. Gene microarray analysis of cultured dysfunctional endothelial cells (induced by treatment with an inhibitor of nitric oxide synthase) revealed upregulation of collagen XVIII, the finding confirmed in vivo in mice chronically treated with NOS inhibitor. Enhanced generation of endostatin in these animals led to the development of endothelial-mesenchymal transition and eventual rarefaction of renal microvasculature, thus further compounding vascular and parenchymal pathology. Endothelial cells exposed to diverse stressors respond with the loss of glycocalyx, lysosomal dysfunction, leakage of cathepsins and degradation of SIRT1, and uncoupling of endothelial nitric oxide synthase. Other components of the extracellular matrix and their effects on regeneration are discussed later in this chapter.

Structural reconditioning

As noted above, cellular structures commonly affected by diverse stressors include mitochondria, lysosomes, and glycocalyx, as well as the extracellular matrix. Those structural aberrations stand on the way of immanent cell or tissue regeneration. The ability to pharmacologically restore such structures may offer therapeutic benefits. A brief analysis of pathways for their restoration and structure-functional consequences of thus conferred reconditioning is provided in this subsection.

- *Lysosomal and mitochondrial pool rejuvenation and replenishment* represent one aspect of cell reconditioning. Examples of such therapies are percolating. For instance, it has been shown that mitochondriopathy of Leigh's syndrome could be corrected using combined mRNA-miRNA nonviral approach to repress mutant T8993G mitochondrial DNA [23]. In addition, a novel mitochondrial-derived, short open-reading-frame of the mitochondrial 12 S rRNA-c encodes a peptide designated as MOTS-c was found to repress folate cycle in the skeletal muscle leading to AMPK activation and improving insulin sensitivity in mouse models of high-fat diet and age-dependent diabetes [24]. It has been proposed that maintaining the level of peroxisome proliferator-activated receptor-γ coactivator 1α or activators of sirtuin-1 can salvage mitochondrial pool and improve nephropathy [25]. Szeto-Schiller peptides have been shown to serve as mitochondrion-protective agents in acute and chronic kidney disease models [9]. On the other hand, it has been advocated that mitochondrial and/or lysosomal transfer via tunneling nanotubes and exosomes may rejuvenate the exhausted pool of these organelles and rescue aerobic respiration [26–28], as illustrated in Fig. 28.2. The corollary of these studies is that a single healthy cell is able to transfer properly functioning organellar cargo during multiple rounds of establishing tunneling nanotubes with cells containing aberrant organelles—a multiplicator effect.
- *Restoration of glycocalyx*—This outermost proteoglycan-rich layer on the surface of cells represents one of the traffic control points for macromolecules, a mechanotransducer from the cell exterior, a gatekeeper for invading cells, a regulator of proliferation, differentiation, adhesion, and motility, as well as coagulation and extracellular antioxidant defense. In the course of diverse kidney diseases, the balance between its synthesis and degradation is shifted toward degradation, thus leading to the net loss of glycocalyx resulting in the loss or perturbation of many functions for which it is responsible. The mechanisms and consequences of glycocalyx loss are described in detail elsewhere [15, 29, 30] and encapsulated in Fig. 28.3. Restoration of glycocalyx, therefore, represents another therapeutic arm of reconditioning, which may restore proper function of diverse aberrant downstream targets.
- *Manipulating cell-matrix adhesion*—Tensegrity, the balance of mechanical forces acting on cells from the extracellular matrix (the outside-in signaling) or from cellular stress fibers and focal adhesions on extracellular

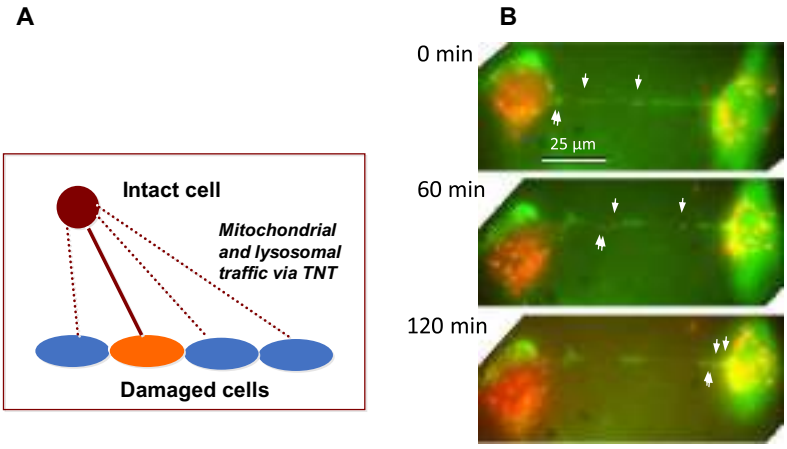

FIG. 28.2 Mitochondrial and lysosomal transfer from intact to damaged cells via tunneling nanotubes (TNT). (A) A schematic view of multiple rounds of TNT transfer. (B) Time-lapse images of lysosomal (*left side* in *red*) traffic from a healthy cultured cell to a cell with lysosomal dysfunction (*right side* in *green*) during 120 min recording interval. Individual lysotracker-labeled lysosomes are marked (*white arrows*). The average speed of lysosomal transfer is 20.7 ± 6.9 µm/h. (Unpublished data obtained by Dr. J Chen in the laboratory.) The similar traffic of mitochondria has been reported by us elsewhere [27].

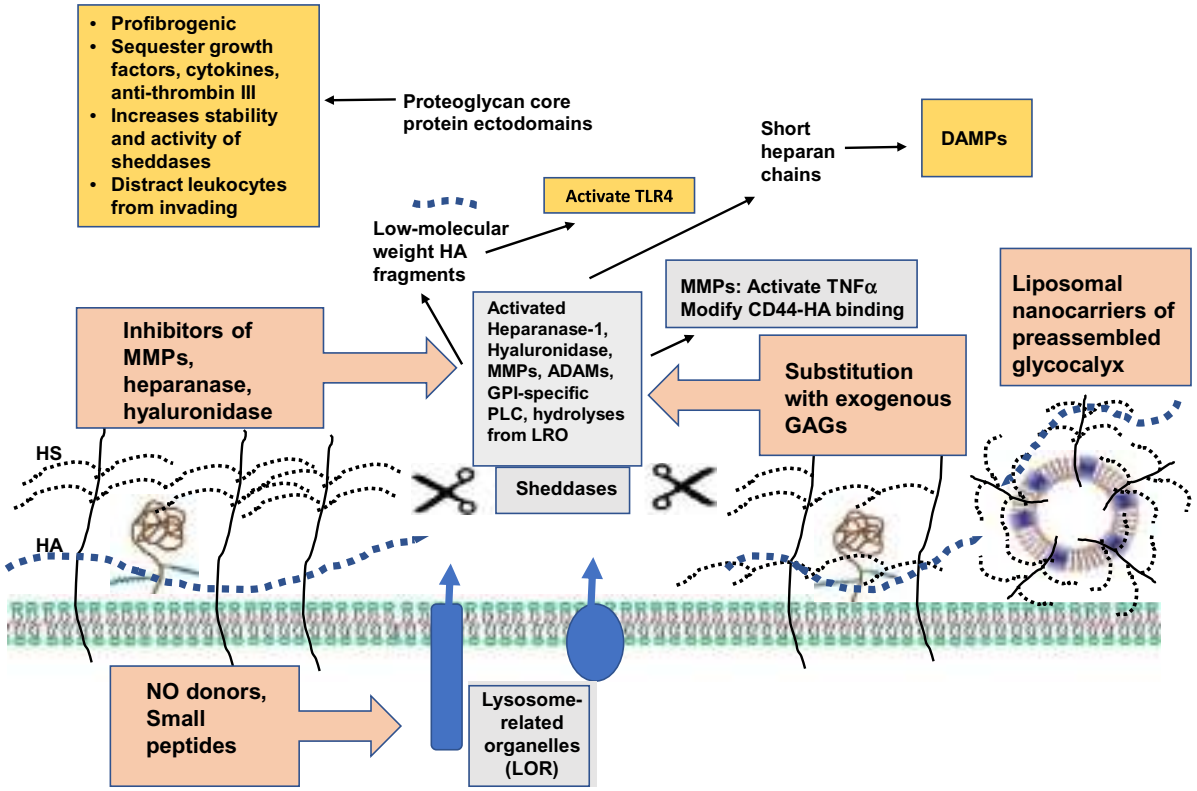

FIG. 28.3 Mechanisms of glycocalyx degradation, pathogenic role of liberated fragments of glycocalyx, pathophysiologic consequences of the loss of endothelial glycocalyx, and groups of compounds proposed to prevent degradation and accelerate restoration of glycocalyx. The pathways of enzymatic degradation of components of glycocalyx are presented in the *central gray box*. Pathogenic features of released fragments of proteoglycan core proteins, short heparan chains, and low-molecular-weight hyaluronic acid are depicted as orange boxes. Diverse families of proposed therapeutics are shown (*pink boxes*). The author has recently proposed the use of liposomal nanocarriers of preassembled glycocalyx, which in the bloodstream hypothetically fuse with the plasma membrane of endothelial and circulating cells to expeditiously restore their lost glycocalyx. Abbreviations: *ADAM*, a disintegrin and metalloproteinase; *DAMP*, danger-associated molecular patterns; *GAG*, glycosaminoglycans; *HA*, hyaluronic acid; *LOR*, lysosome-related organelles; *MMP*, matrix metalloproteinase; *TLR*, Toll-like receptor; *TNF*, tumor necrosis factor.

matrix (the inside-out signaling) are well-known modifiers of cell phenotype [31]. Reprogramming of stem and mature cells can be achieved through changes in their tensegrity. In turn, changes in the rigidity of the extracellular matrix can dictate cell phenotype [32]. Song et al. [33] measured stem cell tensegrity and correlated it with transcription of genes instructive for lineage commitment and confirmed the existence of a significant correlation between live cell stress-strain relations and lineage commitment. Laminar shear stress of 1.5–15 dyne/cm^2 promotes embryonic stem cells to endothelial and hematopoietic differentiation [34]. In mesenchymal stem cells, rigid surfaces induce osteogenic differentiation, while surfaces mimicking the elasticity of muscle induce myogenic differentiation. Similarly, neuronal progenitors differentiate toward neurons on soft matrices, but on rigid ones they acquire glial phenotype. In addition to the rigidity, the topography of the substrate-dependent mechanical forces also plays a role in nuclear reprogramming: culturing fibroblasts on laterally confined microplated surfaces induces acquisition of pluripotency markers even without exogenous reprogramming factors [35]. Comparative analysis of transcriptional responses to shear stress of endothelial colony-forming cells (ECFC) and mature endothelial cells showed that, although they differ under static conditions, shear stress (0.5–2.5 Pa) applied to ECFC leads to expression of differentiation markers similar to those of mature endothelial cells [36]. In this vein, areas with higher rigidity attract different types of cells to adhere, spread, and migrate along the "durotaxis" gradient much stronger than soft substrates. Cell spreading and tight adhesion result in contraction of the matrix, the process that depends on the conformational changes of talin, vinculin, and focal adhesion kinase (FAK) and recruitment of nonmuscle myosin, integrins, and Rac1/RhoA (Ras-related C3 botulinum toxin substrate 1/Ras homolog family member A). In accord with this, inhibition of Rho kinase effector, ROCK, which inactivates myosin, eliminates cell differentiating effects of rigid substrates. Conversion of fibroblasts to myofibroblasts depends on convergence of two signals: one coming from the elevated levels of TGF-β, the other from the stiff matrix with $E > 20$ kPa. In fact, release of TGF-β1, normally sequestered by the extracellular matrix in a latent form as a proTGF-β form, is mediated by αVβ6 integrin binding to the prodomain and liberation of this growth factor from its "straitjacket" of latency-associated protein, together with contraction-induced unfolding of ECM proteins [37]. Mechanical forces also unfold the Notch receptor, thus unmasking the cleavage site and eventually releasing the nucleus-targeting fragment (reviewed in Refs. [38, 39]). In the kidney, as in other organs, injury and inflammation can induce expansion and elevated rigidity of the ECM through its cross-linking, liberation of TGF-β, and further increase in the population of myofibroblasts and deposition of the matrix. This collectively leads to acquisition of profibrotic phenotypic changes. One of the cross-linkers, transglutaminase 2, catalyzes of Ca^{2+}-dependent cross-linking reaction of ECM proteins to stabilize extracellular matrix by forming covalent γ-glutamyl-Nε-lysine bond between acyl donor and acyl acceptor, and scaffolding for cell adhesion induces increase in matrix rigidity [40]. This in turn provides mechanical stimulus for activation of TGF-β, with increased deposition of matrix proteins, thus completing the positive feedback of fibrogenic stimulation [41]. Inhibition of transglutaminase 2 reduces tubulointerstitial fibrosis and preserves function in experimental chronic kidney disease and aging models [41–44]. In fact, there is growing body of work on regulating extracellular matrix stiffness by targeting myofibroblasts. On-going studies encompass miR-29, TGF-β, connective tissue growth factor, lysyl oxidase and tissue transglutaminase, some integrins, growth factor receptors, focal adhesion and Rho-associated kinases, nuclear factor kB, among others, as comprehensively reviewed elsewhere [45].

Another emerging frontier of mechanobiology concerns the transduction of forces generated at focal adhesions to the nuclear envelop and genetic reprogramming [46]. Elevated tensegrity forces lead to cytoskeleton-dependent translocation of normally cytoplasmic YAP (Yes-associated protein), TAZ (transcriptional coactivator Tafazin-associated), and MRTF (myocardin-related transcription factors) to the nucleus, which are important for epithelial-mesenchymal and fibroblast-myofibroblast phenotypic switch (reviewed in Ref. [32]). Lamins adjust nuclear mechanics to the matrix stiffness and tensegrity cytoskeletal forces to further modulate chromatin condensation and remodeling [32]. Thus, the cascade of matrix properties, cytoskeletal tensegrity forces, mechanosensors, and mechanotransducers can regulate nuclear landscape of chromatin condensation, remodeling, and gene expression (Fig. 28.4). This cascade is a plausible target for therapeutic reconditioning and reprogramming [45].

Metabolic reconditioning

Stress-induced metabolic shifts participating in hampering of intrinsic regenerative processes are many, but the most damning among them are represented by key metabolic regulators, as mTOR and sirtuins, metabolic effectors, as nitric oxide synthases, glycolytic and pro-inflammatory gatekeepers. All those and the consequences of metabolic restoration are discussed in the following subsection.

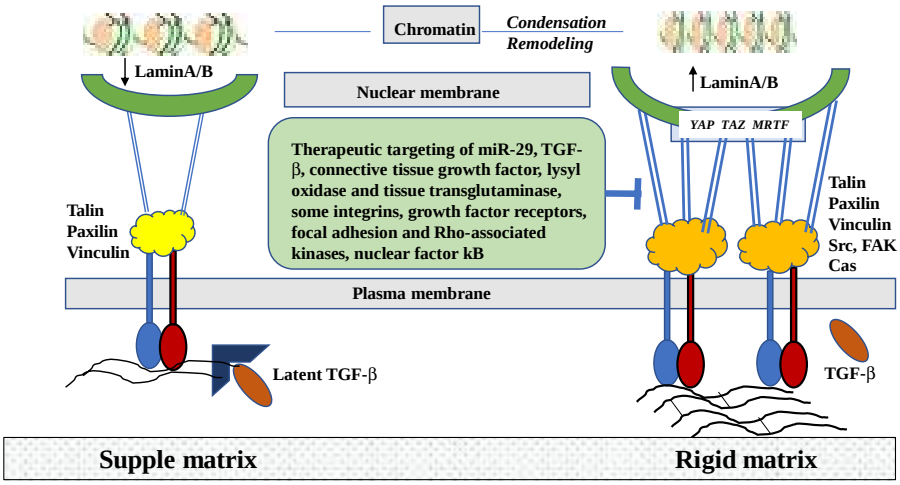

FIG. 28.4 Intracellular mechanosensing and mechanotransduction signaling cascades triggered by extracellular matrix stiffening, like it occurs in unresolved profibrotic conditions. Liberation of TGF-β from its latent form provides reinforcement of matrix-induced signals. Conformational changes in components of focal adhesions generate cytoskeletal tension, which is transmitted to the nucleus to eventuate in changes in gene expression. Selective strategies to ameliorate durotaxis of rigid matrices are presented (*light-green box*).

- *mTOR*—the mechanistic target of rapamycin (mTOR) modulates autophagy. In its active state, mTORC1 mediates anabolic effects, whereas its inactivation signals catabolic states. Notably, activation of mTOR pathway is downstream of insulin and insulin-like growth factor (IGF-1) signaling pathways, which contribute to premature senescence. Targets of mTOR include inhibition of autophagy, regulation of ribosomal biogenesis, protein translation, mitochondrial metabolism, and lipid synthesis. Defective autophagy is responsible for the accumulation of unfolded proteins in the endoplasmic reticulum and induction of unfolded protein response. Antiapoptotic Bcl-2 proteins, often increased in senescent cells, interact with the autophagy protein Beclin1 and inhibit autophagosome formation. Activation of mTOR pathway mediates Wnt/β-catenin pathway [47]. Wnt pathway is chronically activated in a mutant mouse strain deficient in Klotho, a model of premature senescence. Alternative splicing of Klotho gene results in the synthesis of a membrane form of the protein (a coreceptor for FGF23) and a secreted form, which regulates activities of insulin and IGF-1. Recent work identified the retinoic acid-inducible gene-1 (RIG-1) as a target of the intracellular form of Klotho. Moreover, RIG-1 is a potent inducer of interleukins 6 and 8 (IL-6 and IL-8); therefore, defective Klotho signaling reactivates RIG-1 and enhances secretion of IL-6 and IL-8, both pro-inflammatory components of the SASP [48]. mTOR also regulates SASP by promoting IL-1A translation [49].
- *Sirtuins*—The role of SIRT1 in cell fate and metabolic regulation is actuated via deacetylation of approximately 500 target proteins and is based on its effects augmenting FOXO-regulated stress resistance and on its interaction with p53, E2F1, PGC-1a, PPAR-γ, eNOS, NF-κB [50–52], thus regulating cell viability, mitogenesis, proinflammatory, and metabolic pathways. The mTOR and sirtuins pathways are connected via the energy-sensing AMP-activated protein kinase (AMPK), as sirtuin 1 deacetylates liver kinase B1, an activator of AMPK, thus activating it [53]. In turn, AMPK modulates mTOR activity via its upstream regulator TSC2 and Raptor component of mTOR complex 1 [54, 55]. This nexus may represent the point of AMPK interaction with the Wnt pathway, potentially involved in the persistence of profibrogenic and senescent state after an unresolved insult. Notably, mTOR is activated in senescent cells, which show enhanced Wnt signaling, and Wnt9a has been linked to induction of senescence and fibrogenesis in kidneys subjected to ischemia-reperfusion injury [56]. In this context, it is revealing that rapamycin and its analogs, rapalogues, are attracting increasing attention as pharmaceuticals for use in kidney disease, not only in organ transplantation, but also in polycystic kidney disease, renal cell carcinomas, and diabetic nephropathy [57].

It has been well-documented that SIRT1 is a negative regulator of inflammation, in part, due to its effects on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), one of the target proteins for SIRT1 deacetylation [58]. Proinflammatory cytokines induce the activation of the IκB kinase complex, releasing NF-κB from its inhibitors and consequently leading to NF-κB nuclear translocation [52]. SIRT1 binds to and deacetylates p65 protein disabling its transcriptional activity. Accordingly, induction of SIRT1 results in the inhibition of NF-κB-dependent inflammatory pathway and reduced activation of SIRT1 leads to enhanced NF-κB signaling [59]. In turn, the increased NF-κB

signaling hastens the degradation of the endothelial glycocalyx, in part, due to induced shedding of EG proteoglycans and activation of heparanases.

- *Endothelial nitric oxide synthase (eNOS)*—This NO-generating enzyme is present not only in endothelial cells but also in proximal tubules. Recent work has unraveled renoprotective effect of functional eNOS. Zhou et al. [60] demonstrated that multiple actions of NO are mediated via S-nitrosylation of multitude of proteins and specifically, CoA, a central hub for S-NO denitrosylase to transfer SNO to other proteins, thus regulating their functions. SNO-CoA reductase, highly expressed in proximal tubules, opposes this process. In mice with deletion of SNO-CoA-reductase, thus protecting SNO transfer to other proteins, both ischemic and septic insults to the kidney result in a better survival and renoprotection. Metabolic profiling of these kidneys reveals that this effect is mediated by increased SNO inhibitory modification of pyruvate kinase M2, which balances fuel utilization (through glycolysis) with redox protection (through the pentose phosphate shunt) to alleviate oxidative stress. Engagement of these pathways improves resistance to injury, as does knockout of pyruvate kinase M2 in the proximal tubules. Notably, this reversible inhibition (in contrast to irreversible inhibition that promotes diabetic nephropathy) of pyruvate kinase M2 is critical for metabolic reprogramming and kidney preservation in acute kidney injury.
- *Glutamine and glycolysis*—2-D electrophoresis and mass spectrometry analyses of renal microvasculature obtained from mice with chronic suppression of eNOS (a model of global endothelial dysfunction) disclose a perturbed protein profile, including diminished expression of two key enzymes of the Krebs cycle—aconitase-2 and enoyl-CoA hydratase-1, potentially leading to truncation of the Krebs cycle and manifesting in the normoxic inhibition of Krebs cycle and induction of glycolysis—hallmarks of Warburg effect. We hypothesized [61, 62] that supplementation with L-glutamine (GLN), which can enter the Krebs cycle downstream this enzymatic bottleneck, could normalize vascular function and alleviate mitochondrial dysfunction. To test this hypothesis, mice with chronic endothelial dysfunction were treated with glutamine at different concentrations for 2 months. Results confirmed that L-NMMA led to a defect in acetylcholine-induced relaxation of aortic rings that was dose-dependently prevented by glutamine supplementation. Proinflammatory cytokine and chemokine profile in mice with endothelial dysfunction was blunted in mice treated with glutamine. Using an LC/MS platform for metabolite profiling, we identified metabolic perturbations associated with endothelial dysfunction that are offset by glutamine supplementation. Out of 4098 plasma molecules detectable with 100% frequency, 372 were found to be differentially expressed in a four-way comparison of control vs endothelial dysfunction groups, both with and without glutamine. In conclusion, functional and metabolic profiling of animals with early endothelial dysfunction revealed vasculopathy, glutamine deficiency, and showed that supplementation with glutamine is capable to improve vascular function. Whether a similar intracellular glutamine deficiency exists in other cells remains to be established. In this vein, it is remarkable that stimulation of glycolysis (as in glutamine deficiency) promotes reprogramming efficiency in human and mouse fibroblasts and in embryonic stem cells [63, 64]. This is based on acetyl-CoA availability to support histone acetylation. Histone acetylation and open chromatin structure are increased by aerobic glycolysis, whereas acetyl-CoA is consumed in the Krebs cycle, thus resulting in reduced availability of acetyl residues resulting in net chromatin deacetylation, as described earlier in this chapter. It is conceivable that under pathological conditions requiring regeneration, maintaining the balance of acetylation-deacetylation through judicious stimulation and inhibition of reprogramming may exert therapeutic effects.
- *NF-κB pathway*—SASP, a portion of cell secretomes, is perpetrated by induction of NF-κB transcription factor and CCAAT enhancer binding protein β [65]. On the other hand, interleukin-1α, one of the SASP products, exerts positive feedback on NF-κB and suppression of Klotho expression to enhance SASP release [66]. The general facts on NF-κB transcriptional activity dependence on sirtuin 1 were summarized above. We studied mice with endothelial sirtuin 1 devoid of deacetylating activity ($SIRT1^{endo\ -/-}$ mice) and observed a dramatic increase in syndecan 4 in the secretome of TGF-β-activated microvascular endothelial cells isolated from kidneys. Remarkably, promoter regions of both SIRT 1 and syndecan 4 harbor NF-κB-response element [52]. Despite the upregulation of syndecan 4, a major proteoglycan component of endothelial glycocalyx and a binding partner of glycosaminoglycans comprising this structure, the expression of glycocalyx is dramatically reduced in $Sirt1^{endo\ -/-}$ mice. Data obtained through western blots of decellularized kidney matrix of unilaterally obstructed kidneys reveal a decrease of the whole Synd4 molecule with an increase in a syndecan 4 fragment, a result of enhanced syndecan 4 shedding. The ectodomain alone can promote fibrogenesis [67]. These studies reveal a novel consequence of SIRT1 deficiency—loss of endothelial glycocalyx.

Pharmacologic reconditioning

Remarkably, several well-established and a few novel compounds can be repurposed for the goals of in vivo cell reconditioning. Although these compounds are mechanistically interrelated, the following groups are emerging.

- NAD^+—Nicotinamide mononucleotide and the nicotinic acid represent vitamin B3 or niacin. Vitamin B3 deficiency known as pellagra manifests in the "3Ds"—diarrhea, dermatitis, and dementia. Supplemental NAD^+ precursor nicotinamide mononucleotide restores vascular dysfunction in aging mice [52]. The de novo synthesis from tryptophan can be impaired in Hartnup disease due to the defect in intestinal absorption or in carcinoid syndrome associated with massive production of serotonin, a synthetic product of tryptophan. Pharmacologic supplementation with nicotinamide mononucleotide (converted to NAD^+ by nicotinamide phosphoribosyltransferase) or nicotinamide riboside (converted to NAD^+ by two nicotinamide riboside kinases), as well as other NAD^+ precursors represent a well-established target to improve sirtuins activity and, with it, recondition metabolism for improved regenerative capacity. This can be accomplished together with the growing numbers and improving efficacy of sirtuin-activating compounds [68].
- Rapalogues—Based on the effects of mTOR complex 1 on HIF-1α, NF-κB, autophagy, lipogenesis, ribosomal biogenesis, and translational activity, the growing family of rapamycin analogs is gaining in recognition as potential rejuvenating agents, as detailed in Chapter 30.
- Activators of eNOS and NO donors—Therapeutic interventions leading to enhancement of eNOS function are emerging. The use of an eNOS enhancer AVE9488 may offer potential means to restore NO bioavailability [69, 70].
- Senomorphs to control SASP—A recently disclosed action of metformin in activating AMPK has been attributed to its senomorphic effect. The similar SASP-reducing senomorphic effect is afforded by rapamycin. Activation of sirtuins using resveratrol, as an example of rejuvenating strategy that also reduces oxidative stress and ameliorates pro-inflammatory components of SASP, has shown experimental benefits (reviewed in Ref. [71]). More recently, a number of small molecule sirtuin 1 (SIRT1) activators (STAC) has been synthesized [72], all extending life- and health-span and presently undergoing clinical trials. Another compound emerging in this category is NAD^+, a cofactor necessary for activation of several sirtuins. NAD^+ bioavailability is reduced in disease states and aging [16] and its precursor, nicotinamide, is therapeutic in correcting NAD^+ deficiency.

The road ahead

As the wave of regenerative medicine engulfs new grounds and attracts, in addition to transplant surgeons, molecular and cellular biologists, bioengineers, and epigeneticists, a new rivulet of cellular reconditioning is springing. Ideas of cellular reconditioning rest on the premise that in the course of diverse diseases defaulting organelles, metabolic pathways and microenvironmental cues derail innate regenerative processes. This premise is starting to gain experimental support with recognition of intrinsic proregenerative forces impeded in the course of disease and explains the emergence of strategies to tinker with dysfunctional organelles, aberrant intra- and extracellular metabolites, and nonpropitious for restitution cell-matrix adhesion. Specifically, further developments in chemical and bioengineering designs to restore dysfunctional organelles such as mitochondria, glycocalyx, and other are required. There are palpable advances in modulating cell-matrix adhesion, but the climb to viable therapeutics remains steep. Deeper understanding of metabolic derangements that hinder regeneration is needed to correct these abnormalities. The balance between the innate regeneration and forced regeneration remains tenuous. Undoubtedly, this fledgling field of regenerative sciences is beckoning us for experimental and conceptual buttresses.

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CHAPTER

29

Senomorphic, senolytic, and rejuvenation therapies

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Introduction

Cellular senescence is a cell cycle arrest status with cellular phenotypic alterations, including changes in apoptotic pathways and pro-inflammatory secretome [1]. Senescence can be caused in vitro by various types of stress, such as radiation, oncogene activation, chemotherapy, reactive oxygen species (ROS), and DNA damage [2–4]. In renal diseases, stressors have been hypothesized to involve in vivo inducers of senescence, including urinary toxins, excessive activation of renin angiotensin aldosterone system (RAAS), DNA damage, reactive metabolites, ischemia/reperfusion injury, inflammation, epigenetic stress, and damage-associated molecular pattern proteins (DAMPs) [5–10] (Fig. 29.1). As described in other chapters in this book, major pathways involved in senescence are the p53/p21 and p16 tumor suppressor senescence effector pathways. While moderate and temporary p16 expression has beneficial effects in restraining unnecessary cell proliferation [11], in severe stress, upregulated p16 expression becomes irreversible, resulting in a permanent senescent cell growth arrest. Senescent cells have heightened survival and metabolic activity, making them resistant to apoptosis. Resistance to apoptosis in senescent cells is driven by upregulating several senescent cell antiapoptotic pathways (SCAPs), include B-cell lymphoma 2 family inhibitors (BCL)-2, PI3K/Akt pathways, p53/p21 pathways, hypoxia inducible factor (HIF)-1α, and heat shock protein (HSP-90) [2, 4, 11]. These networks and other emerging SCAPs led to the initial discovery and development of the first class of senolytic drugs, which attempt to remove senescent cells [2, 4, 11]. In addition, senescence also activates production and secretion of cytokines, chemokines, and extracellular matrix degrading proteins, termed the senescence-associated secretory phenotype (SASP) [4, 11, 12]. Regulators of the SASP in senescent cells include nuclear factor (NF)-κB, CCAAT-enhancer-binding proteins (C/EBP), interleukin (IL)-1α, IL-6, etc. (Fig. 29.1). The inflammatory cytokines from the SASP may attract immune cells to tissue injury sites in order to initiate the natural processes of wound healing and tissue regeneration.

In contrast to these short-term beneficial biological functions, senescent cells may accumulate in excess chronically at sites of injury, where old tissues or pathology such as diabetes may drive age-related tissue dysfunction in acute and chronic diseases. For example, there is a strong connection between acute kidney injury (AKI) and mechanisms of senescence activation [13]. Thus, clearance of senescent cells has been promoted as a novel therapeutic strategy to treat renal diseases. One approach has been to prevent senescence by limiting triggers inducing senescence through lifestyle and antiaging drugs, such as caloric restriction or antioxidant agents. Caloric restriction has been proven to have life-extending effects in rodents, and antioxidants might be beneficial by reducing ROS-mediated DNA damage. However, elimination and attenuation of cellular senescence require specific targeting. This chapter will focus on such senotherapeutic approaches, including drugs that selectively eliminate senescent cells ("senolytics"), or those that inhibit the SASP ("senomorphics") in renal diseases. Drugs and compounds that have been used in antisenescence research are summarized in Table 29.1.

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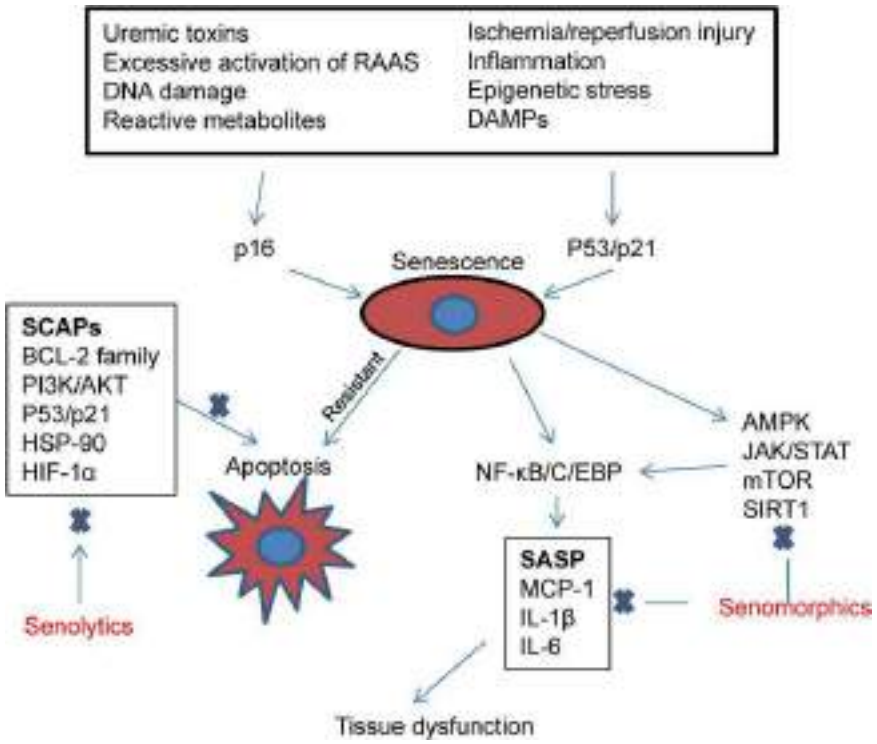

FIG. 29.1 A summary of the common causes, involved pathways, and senotherapies in renal diseases. *AMPK*, AMP-activated protein kinase; *BCL*, B cell lymphoma 2 family inhibitors; *C/EBP*, CCAAT-enhancer-binding proteins; *DAMPs*, damage-associated molecular pattern proteins; *HSP-90*, heat shock protein 90; *HIF*, hypoxia inducible factor-1α; *IL (interleukin)-1α*, IL (interleukin)-6; *JAK*, Janus-associated kinase; *MCP*, monocyte chemoattractant protein-1; *mTOR*, mechanistic target of rapamycin; *NF*, nuclear factor-κB; *RAAS*, renin angiotensin aldosterone system; *SASP*, senescence-associated secretory phenotype; *SCAPs*, senescent cell anti-apoptotic pathways; *SIRT*, sirtuin.

TABLE 29.1 Drugs and compounds have been used in anti-senescence research.

Drugs/compounds	Mechanisms	Targeting sites
Dasatinib	Senolysis	PI3K/AKT
Fisetin	Senolysis	PI3K/AKT
Quercetin	Senolysis	PI3K/AKT, p53
Alvespimycin	Senolysis	HSP90
Cardiac glycosides	Senolysis	Na^+/K^+-ATPase
ABT-737	Senolysis	BCL-2
ABT-263	Senolysis	BCL-2
A1331852	Senolysis	BCL-2
A1155463	Senolysis	BCL-2
Piperlongumine	Senolysis	Caspase-3
UBX0101	Senolysis	p53
FOXO4-DPI	Senolysis	FOXO4
Apigenin	Anti-SASP	NF-κB
Wogonin	Anti-SASP	NF-κB
Kaempferol	Anti-SASP	NF-κB
Resveratrol	Anti-SASP	NF-κB
Metformin	Anti-SASP	NF-κB

TABLE 29.1 Drugs and compounds have been used in anti-senescence research—cont'd

Drugs/compounds	Mechanisms	Targeting sites
SB203580	Anti-SASP	MARK/NF-κB
UR-13756	Anti-SASP	MARK/NF-κB
BIRB796	Anti-SASP	MARK/NF-κB
PF-3644022	Anti-SASP	MARK/NF-κB
MK2.III	Anti-SASP	MARK/NF-κB
Rapamycin	Anti-SASP	mTOR/NF-κB
Rapalogs	Anti-SASP	mTOR/NF-κB
Ruxolitinib	Anti-SASP	JAK/IL-6
Tofacitinib	Anti-SASP	JAK/IL-6
Canakinumab	Anti-SASP	IL-1β

BCL, B cell lymphoma 2 family inhibitors; *HSP-90*, heat shock protein 90; *IL (interleukin)-1α*, IL (interleukin)-6; *JAK*, Janus-associated kinase; *MAPK*, mitogen-activated protein kinase; *mTOR*, mechanistic target of rapamycin; *NF*, nuclear factor-κB; *SASP*, senescence-associated secretory phenotype.

Senomorphic drugs

There are several options to modulate SASP by targeting different intracellular pro-inflammatory signaling pathways without necessarily inducing apoptosis in senescent cells. These include strategies targeting NF-κB/C/EBP, Janus-associated kinase (JAK)/STAT, AMP-activated protein kinase (AMPK), mechanistic (previously referred to as mammalian) target of rapamycin (mTOR), and sirtuin (SIRT1) pathways. Several well-established senomorphic and senescence-preventing strategies are discussed below.

NF-κB/C/EBP

The NF-κB pathway is closely linked to Klotho. Studies in ischemia reperfusion injury (IRI) have demonstrated downregulated expression of Klotho, which possesses renoprotective properties capable of attenuating of kidney injury. Zeng et al. [14] employed senescence-accelerated mice (SAMP8) and their corresponding normal control SAM resistant-1 (SAMR1) to investigate the potential role of Klotho in aging-associated inflammation and renal injury. They identified early-stage chronic kidney disease (CKD) in aged SAMP8 mice compared with aged SAMR1 controls, manifesting in an increase in the urinary albumin-to-creatinine ratio, inflammatory cell infiltration, glomerulosclerosis, and tubulointerstitial fibrosis. The loss of Klotho led to activation of the NF-κB signaling pathway, and subsequent production of tumor necrosis factor (TNF)-alpha and IL-6 in the kidneys of aged SAMP8 mice. These finding suggested Klotho and NF-κB as potential therapeutic targets in aging-related inflammation and development of early-stage CKD.

In patients with kidney failure, uremia may induce endothelial cell senescence, which can lead to endothelial dysfunction and cardiovascular disease. In vitro exposure to uremic serum led to telomere length shortening, increased beta-galactosidase and oxidative stress, as well as NF-κB activity in endothelial cells. The expression of Klotho decreases in uremic serum-treated cells and exogenous Klotho prevents cellular senescence by inhibiting the NF-κB-DNA binding ability [15]. Furthermore, indoxyl sulfate, a uremic toxin, downregulates renal expression of Klotho through production of ROS and activation of NF-κB [16].

Several animal studies have tested agents that inhibit NF-κB activation in aged kidneys. Kim et al. [17] found that baicalin, a flavonoid isolated from Scutellaria baicalensis, suppresses age-related NF-κB activation in kidney tissue from old rats, and Lim et al. showed that this is mediated through PPARγ activation [18]. Park et al. [19] observed the antiinflammatory effect of kaempferol in aged kidney tissues via nuclear factor-inducing kinase/IκB kinase. Kim et al. [20] confirmed that kaempferol modulates NF-κB activation by suppressing advanced glycation end products (AGE)-induced upregulation of NADPH oxidase. C/EBP interaction with NF-κB p50 homodimer provides an alternative pathway for NF-κB target gene activation, and this interaction may enhance gene activation [21]. Studies [22] also suggest that C/EBPα knockout in podocytes aggravates podocyte senescence through the AMPK/mTOR

pathway, leading to glomerulosclerosis, and that subsequent albuminuria exacerbates the epithelial-mesenchymal transdifferentiation of senescent tubular cells by suppressing autophagy. These studies highlight the importance of NF-κB/C/EBP as a new potential target for SASP inhibition in kidney aging and diseases.

JAK inhibitor

Ruxolitinib is an orally bioavailable selective inhibitor of JAK1/2. In unilateral ureteral obstruction (UUO) mice [23], ruxolitinib alleviated UUO-induced inflammation, oxidative stress, and apoptosis. Kidneys from UUO mice with ruxolitinib treatment displayed less tubular injuries and renal fibrosis. A case report [24] also showed significant improvement in glomerulopathy in patients with primary myelofibrosis treated with ruxolitinib. Strati et al. [25] performed a retrospective study in 100 patients with previously untreated primary myelofibrosis, receiving a single-agent ruxolitinib, compared to 105 patients receiving with a nonruxolitinib-based therapy, matched by age, sex, and estimated glomerular filtration rate (eGFR). Their results showed that use of ruxolitinib was associated with a significantly higher rate of renal improvement. Inhibition of the JAK pathway suppresses the SASP in senescent cells and thus alleviates age-related tissue dysfunction [26]. Patients with myelofibrosis with varying degrees of CKD can derive meaningful benefit from ruxolitinib even at a low dose [27], but more clinical trials are needed for ruxolitinib as a standard SASP inhibition treatment in patients with renal dysfunction.

AMPK pathway

The best-known agent that affects the AMPK pathway is metformin, a biguanide used for treatment of type 2 diabetes, and which also shows promise as an antiaging agent. The Food and Drug Administration revised guidelines, now allowing patients with diabetes with mild to moderate impairment in kidney function to be treated with metformin. Metformin [28] is the first drug to be tested for its age-targeting effects in the large clinical trial-TAME (Targeting Aging by MEtformin). Metformin attenuates hallmarks of aging by improving nutrient sensing, enhancing autophagy, delaying stem cell aging, modulating mitochondrial function, regulating transcription, and lowering telomere attrition and senescence. These salutary characteristics make metformin an attractive drug to translate to human trials, a very promising step given its renal protective abilities in patients with diabetic and even nondiabetic kidney disease. Metformin may prevent inflammatory responses through decreasing immune cell infiltration into renal tissue in UUO [29] or cisplatin-induced AKI [30], thereby reducing inflammatory damage. Metformin also reduces mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in AGEs-treated bone-marrow-derived macrophages via the AMPK-NFκB pathway [31], as well as in the renal tissue of the UUO model [32]. Metformin may directly inhibit NFκB activation and generation of pro-inflammatory SASP cytokines, such as monocyte chemoattractant protein-1, intercellular cell adhesion molecule-1, and transforming growth factor (TGF)-β1 in high glucose-treated rat glomerular mesangial cells in vitro [33]. Metformin may also interfere with AMPK-independent pro-inflammatory pathways as an antiaging agent [28]. Alfaras et al. [34] examined the effects of 1% metformin given every-other week on survival of 2-year-old male mice fed standard chow. Metformin showed benefits on metabolic markers, although age-associated kidney lesions became more pronounced but without pathological consequences. The renal effects of metformin on a broad range of renal diseases have been recently reviewed by Pan et al. [35]. The clinical efficacy of metformin needs to be validated in well-designed randomized controlled trials with larger sample sizes and longer duration.

mTOR inhibition

Rapamycin is a clinically approved mTOR inhibitor that can stimulate intracellular repair and autophagy. The use of mTOR inhibitors in renal diseases has been critically reviewed [36]. Rapamycin can decrease DNA damage in vivo and rescue shelter in gene expression [37]. In animal studies, rapamycin shows beneficial effects by reducing UUO-induced renal hypoxia, inflammation, and tubulointerstitial fibrosis [38, 39]. The upregulated mTORC1 activity in renal glomerular and tubular epithelia from both transplant and native biopsies indicates that the activated mTOR signaling is a general phenomenon in variants of progressing renal diseases and may be used as a marker of active renal disease states [40]. Inhibition of mTOR leads to changes in peritubular endothelial cell phenotype, indicating renal protection [41]. Clinically, rapamycin is often prescribed as an immunosuppressive drug to prevent rejection after kidney transplantation and modulates Klotho expression through mTORC2 activation [42]. Rapamycin and its analogs [43] may increase life span and delay age-related phenotypes in many species by inhibition of mTORC1. Interestingly, SIRT1 seems to be required for the effects of rapamycin on high glucose-inducing mesangial cells senescence [44]. However, side effects of rapamycin include insulin resistance, glomerular dysfunction, dyslipidemia, anemia, leucopenia, and

thrombocytopenia, mucositis, pneumonitis, lymphedema, angioedema, and osteonecrosis. This wide variety of side effects likely reflects the broad involvement of the mTOR pathway in cell and tissue homeostasis and may limit its use.

SIRT1 activators

Another option to reduce the SASP is via SIRT1 activation. SIRT1 not only inhibits NF-κB signaling but also alleviate cell cycle arrest by regulation of p53. Sirt1-induced deacetylation of HIF-1 alpha may have protective effects against tubulointerstitial damage in the aged kidney [45]. Pharmacological targeting of SIRT1/Nrf2 signaling molecules may reduce the pathologic changes of aging in the kidney [46, 47]. Increased sirtuin activation can be achieved via dietary supplementation of NAD + with niacin [48, 49] or with resveratrol. In vitro, resveratrol protects against high-glucose-triggered oxidative stress and cell senescence. Moreover, a 3-month chronic treatment with resveratrol prevents high-fat diet-induced renal pathological damage by activating SIRT1 [50]. Resveratrol protects diseased kidneys also through upregulation of antiaging Klotho gene expression via the activating transcription factor 3/c-Jun complex-mediated signaling pathway [51], or by reducing oxidative stress, inflammation, and fibrosis, through Ang II suppression [52]. Therefore, modulation of the SIRT1 pathways offers several venues for attenuation of senescence.

Although SASP modulation is expected to limit detrimental paracrine effects of a prolonged exposure to senescent cells, it could also have harmful side effects. Because SASP factors are often not senescence-specific but are upregulated in a broad spectrum of different pathways, any intervention could thus interfere with vital processes. Furthermore, SASP factor modulation could impede immune surveillance and hamper elimination of senescent cells and should thus be employed cautiously.

Senolytic drugs

Compared with the chronic treatment for attenuation of SASP, senolytics approaches to remove senescent cells may be the most feasible and attractive strategies for clinical application. Senolytic agents are a class of small molecules that can selectively kill senescent cells that participate in senescence-associated pathways by interfering with their anti and prosurvival signaling [53]. Senescent cells have much in common with cancer cells, including similarities in metabolic activity. Therefore, comparable strategies used for triggering apoptosis in cancer cells are being explored for selective removal of senescent cells, and antitumor drugs are investigated for their potential as senolytic agents. Importantly, several senolytic drugs are already in clinical use or in advanced phases of antitumor drug development, and the use of existing antitumor drugs as senolytics has gained interest. Although very few of these drugs have been tested in renal diseases, many have already been shown to be useful in a variety of cell types and tissues both in vivo and in vitro, suggesting a potential for application in kidney diseases as well. Below, we summarized the effects of some candidate senolytic drugs with clinical potential according to their targeting pathways.

BCL-2 family

The inhibition of the interaction of BCL-2 family protein with BAX and BAK enables them to form oligomers and induce apoptosis. Several agents target different BCL-2 family proteins. A promising pan-BCL inhibitor, navitoclax (ABT-263), imposes a senolytic effect on different human and mouse cells, including renal epithelial cells [54, 55]. Navitoclax clears senescent cells and reduces SASP in old and irradiated mice. Furthermore, treatment of mice with navitoclax results in improved myeloid function and reversed pulmonary fibrosis [55]. However, in human mesenchymal stem cells (MSCs), navitoclax showed only low selectivity for senescent cells compared to nonsenescent cells, and none was observed in human preadipocytes [54]. Another successful BCL-2 inhibitor, venetoclax (ABT-199), has been FDA-approved for treatment of refractory chronic lymphocytic leukemia [56]. Although successful in oncology, the in vitro senolytic effect of venetoclax in human fibroblasts is variable and dependent on the manner of senescence induction [55, 57]. Evidently, this class of drugs may be suitable only for selected cell types.

PI3K/AKT

Quercetin

Quercetin belongs to the polyphenols and is subclassified as a flavonoid, commonly used as an antioxidant. Interestingly, quercetin also activates Sirt1-mediated deacetylation of p53, improving mitochondrial function and decreasing NF-κB, resulting in decreased fibrogenesis [4, 58]. We have recently reported [59] that C57BL/6J mice fed

with high-fat diet developed obesity and hypercholesterolemia, which impaired renal function and cortical oxygenation, and induced glomerulomegaly. Renal markers of senescence (expression of p16, p19, and p53) and its secretory phenotype were upregulated, but were all attenuated after treatment with quercetin (50 mg/kg) 5 days biweekly via oral gavage for 10 weeks.

To extend the therapeutic range, quercetin has often been combined with the tyrosine kinase inhibitor dasatinib. In vitro, this combination selectively reduces senescent human endothelial cells and mouse fibroblasts but is less effective in preadipocytes and human lung fibroblasts [2, 53]. Treatment with quercetin and dasatinib of primary human adipose tissue from obese patients resulted in decreased expression of senescence markers and reduced SASP production [58]. Quercetin and the quercetin-dasatinib combination have also been applied in several mouse models, including naturally aged and accelerated aging mice, obesity-induced metabolic dysfunction, single-leg radiation, atherosclerosis model, liver steatosis, bleomycin-induced pulmonary injury, and transplantation of senescent cells. Combined treatment with quercetin and dasatinib of mice with metabolic syndrome not only resulted in improved glucose metabolism but also in decreased albuminuria. In naturally aged mice, quercetin and dasatinib decreased cell senescence in fat and liver, improved left ventricular ejection fraction, decreased senescence in endothelial and smooth muscle layers, diminished physical dysfunction, reduced SASP expression, and improved bone microarchitecture [58]. Treatment of mice results in a remarkable improvement of several aging-related parameters, such as muscle function and coordination [53].

Moreover, this combination is already employed in clinical trials. In an open label Phase 1 pilot study [60], Hickson and colleagues administered 3 days of oral dasatinib (100 mg) and quercetin (1000 mg) to subjects with diabetic kidney disease. This combination reduced adipose tissue senescent cell burden within 11 days, with decreases in p16(INK4A)-and p21(CIP1)-expressing cells, cells with senescence-associated beta-galactosidase activity, and adipocyte progenitors with limited replicative potential. Treatment also reduced adipose tissue macrophages, which are attracted, anchored, and activated by senescent cells, as well as crown-like structures, representing adipose inflammation. Skin epidermal p16(INK4A +) and p21(CIP1 +) cells were reduced, as were circulating SASP factors, including IL-1alpha, IL-6, and MMPs-9 and -12. These exciting data showed that the combination of dasatinib and quercetin significantly decreases adipose tissue senescent cell burden in humans. Further studies are needed to determine their effects on the human kidney.

Fisetin

Fisetin, another member of the flavonoid family, targets a plethora of signaling pathways, including PI3K/AKT, NF-κB, p38 MAPK, and BCL-2/BCL-XL [61]. Fisetin has been shown to be beneficial in cisplatin-induced AKI [62]. In vitro, fisetin reduces etoposide-induced senescent cells in human and mouse cells by alleviating the cell cycle arrest [63]. In aging mice (22–24 months), fisetin treatment in vivo decreased cellular senescence in kidney, liver, fat, and spleen, and reduced levels of circulating SASP factors [63]. Given its good safety profile, additional studies are needed to determine its efficacy.

FOXO4

The FOXO4 D-retro inverso (DRI) peptide targets the FOXO4-p53 axis, leading to an increase in active p53 in senescent cells. In a mouse model of folic-acid-induced kidney damage, FOXO4 DRI significantly reduced senescent tubular cells, but not renal fibrosis [64]. On the other hand, in a mouse model of accelerated aging, FOXO4 DRI treatment was associated with preserved kidney function [2], and in human fibroblasts induced a selective reduction of senescent compared to nonsenescent cells [2].

Heat shock protein 90

HSP90 is a molecular chaperone that is especially transcribed during cellular stress, aiding protein stabilization and preventing protein misfolding and aggregation. HSP90 client proteins include p53, eNOS, TERT, and mitochondrial proteins. HSP90 has an antiapoptotic effect mediated by mTOR, NF-κB, and FOXO3A and leads to AKT stabilization mitigating apoptosis [65]. Thus, inhibition of HSP90 has senolytic potential. Several HSP90 inhibitors such as 17-DMAG and geldanamycin showed strong senolytic effect in senescent human MSCs and fibroblasts [65]. Furthermore, progeroid mice treated with 17-DMAG showed reduced age-related symptoms and increased overall condition [65]. HSP90 inhibitor partially corrects nephrogenic diabetes insipidus in a conditional knock-in mouse model of aquaporin-2 mutation. However, its specificity for senescent cells needs to be determined, given that nonselective apoptosis may result in elimination of otherwise useful cells.

In general, while senolytic therapy is potentially beneficial in reversing age-related diseases, off-target effects might limit their use. For instance, several potent senolytic agents, such as navitoclax and ABT-737, show major side effects like thrombocytopenia and neutropenia when administered systemically. Targeted delivery of senolytic compounds to senescent renal cells may allow to decrease systemic exposure and toxicity. Targeted accumulation of senolytic agents in the kidney might be achieved using nanoparticulate carriers, such as conjugates and liposomes. The delivery of therapeutic of such functionalized compounds may enable high enough drug concentrations where needed. Major methods for targeting proximal renal tubular cells involve protein- or peptide-based carriers, or the use of nanoparticles [66]. Protein- or peptide-based carriers consist of a low-molecular-weight protein to which the drug is linked [67]. Further in vivo targeting delivery studies are needed in both preclinical and human settings to determine their safety, specificity, and efficacy.

Renal rejuvenation

The kidney has limited regenerative capacity. Kidney tubules might be regenerated after injury, with origin of regenerating cells being stem cells localized in the papilla or from proliferation of local cells [68]. We have reported [69] that intrinsic renal scattered tubular cells (STC) contribute to repairing injured tubular epithelial cells by releasing extracellular vesicle (EV). However, we also found that STC function is impaired in kidney diseases [70, 71], and the lack of effective treatment to prevent progression to end-stage kidney failure creates an important impetus to develop regenerative cell-based therapies for CKD utilizing exogenous cell delivery.

The most commonly applied cell types in renal regeneration have been endothelial progenitor cells (EPC) or MSC, as described in other chapters in this book. EPCs are progenitor cells expressing CD34 and KDR, which originate from bone-marrow-derived hematopoietic stem cells, patrol the systemic circulation, and contribute to repair where needed. Their mobilization from bone marrow to the peripheral circulation is highly regulated under both normal physiological conditions and stress. EPCs that are mobilized and recruited to the injured tissue contribute [72] to neovascularization and tissue repair in the musculoskeletal, neural tissues, and the bone. Our previous studies [73, 74] also showed that exogenous expanded EPC can home to injured kidney and restore renal function in chronic experimental renovascular disease. MSCs are cells that express CD44, CD73, and CD90, derived from different adult (adipose tissue, peripheral blood, bone marrow) and neonatal tissues (particular parts of the placenta and umbilical cord), which have greater availability than EPC. MSCs protect injured kidneys [75, 76] through multiple effects, including proangiogenic, antiinflammatory, and antifibrotic activity by paracrine mechanisms, which may be mediated through different pathways compared with EPC [77]. However, harvesting a large amount of cells for therapeutic uses requires in vitro expanding for both EPC and MSC by repeat passaging, which may cause cells loss of stemness and increasing senescence. MSCs expanded in vitro develop senescence after several passages and gradually lose the typical fibroblast-like spindle shape, leading to morphological abnormalities and inhomogeneity [78]. These are accompanied by decreased CD146 expression and by genetic instability.

Effects of renal diseases on stem cell function

In CKD, multiple factors may impair intrinsic renal stem cell function, as well as EPC and MSC function, including aging, coexisting cardiovascular disease, diabetes, and uremia, which may limit the effectiveness of an autologous cell-based therapy approach [79]. Some of these adverse effects are caused by increased senescence of reparative cells, which hinders their normal repair function. Klinkhammer [80] investigated the effects of remnant kidney (RK)-induced CKD in rats on bone-marrow-derived MSC function in vivo and in vitro. Under this condition, MSCs showed signs of premature senescence: spontaneous adipogenesis, reduced proliferation capacity, active senescence-associated-beta-galactosidase, accumulation of actin, and a modulated secretion profile. Furthermore, CKD-RK-MSC exerted no benefit on injured kidneys compared to healthy MSCs, which reduced accelerated scarring of glomerular lesions. Noh et al. [81] also investigated MSCs function in uremia induced by partial kidney ablation in C57Bl/6J mice. Uremic MSCs showed increased cellular senescence, decreased proliferation, deficient migration capacity, and impaired tube formation. A diminished mitogenic effect on endothelial proliferation was also observed in conditioned media collected from uremic MSCs, and intravital microscopic analysis showed decreased angiogenesis potency in uremic MSCs. Thus, in rodents CKD leads to a sustained loss of in vitro and in vivo functionality of MSCs, possibly due to premature cellular senescence.

In human subjects [82], functions of MSCs obtained from the adipose tissues of patients undergoing long-term dialysis (KD-MSCs) were compared from healthy controls (HC-MSCs). Interestingly, no significant changes in

proliferation potential, senescence, or differentiation capacity were observed in KD-MSCs. However, a directed in vivo angiogenesis assay revealed a decrease in angiogenic capacity in KD-MSCs, and their response to hypoxia was blunted. Wang et al. [83] further demonstrated that indoxyl sulfate, a uremic toxin, induced cellular senescence in human umbilical cord-MSCs, and their ability to stimulate CD4 + CD25 + FoxP3 + regulatory T-cell production was compromised, indicating impaired immune-modulation function. Contrarily, another study [84] reported that MSCs' intrinsic capacity to produce proangiogenic paracrine factors, including their extracellular vesicles (EVs), is not affected by donor CKD. Likely, factors such as severity, duration, and comorbidities in CKD determine stem cell function, as does the specific functional attribute studied.

Metabolic alterations also impair MSC function in vitro. Using a porcine model of obesity and metabolic syndrome (Mets), we [85] compared RNA and protein profiles in MSC isolated from lean and Mets animals. MetS-MSCs exhibited increased nuclear translocation of nuclear factor kappa-B, associated with increased SA-beta-galactosidase. In human subjects [86], we also found that obesity triggers an early senescence program in adipose-tissue-derived MSC. MSC from obese subjects exhibited lower proliferative capacities than nonobese-MSC, suggesting decreased function. Senescent cell burden and phenotype, manifested as p16, p53, IL-6, and MCP-1 gene expression, were upregulated in obese subjects' MSC. Furthermore, coincubation with nonobese, but not with obese-MSC, restored function to injured HUVEC. Thus, obesity-induced cellular injury may alter efficacy of this endogenous repair system and hamper the feasibility of autologous transplantation in obese individuals.

Many factors may affect the bone marrow and thereby impair bone marrow stromal cells and EPC function, although this has not been as extensively studied as on MSC. For example [87], bone marrow stromal cells harvested from Klotho null mice developed fewer osteoblastic but more adipocytic colonies than cells from wild-type mice. Klotho was weakly expressed and FGF23 dose-dependently affected the lineage fate determination. As mentioned previously, decreased Klotho expression often accounts for cell senescence. Indeed, Klotho-overexpressing bone marrow MSCs have increased proliferative ability and more potent immunoregulation ability [88].

Interestingly, young stem cells isolated from umbilical cord are more potent than aged adipose-tissue-derived stem cells for reducing the incidence of the senescent phenotype in the kidneys [89]. Treatment with human umbilical-cord-derived MSCs ameliorated long-term kidney function after ischemia reperfusion injury, attenuated renal fibrosis, decreased beta-galactosidase expression, and increased the expression of Klotho, while adipose-derived MSCs improved renal function to a lesser degree and achieved a less pronounced increase in the renal expression of Klotho and manganese superoxide dismutase. Furthermore, Fabian et al. [90] demonstrated in vivo that MSC biodistribution posttransplantation is detrimentally affected by aging of both the recipient and the donor MSCs, which attenuates transplantation efficiency. It is likely that aged MSCs should be rejuvenated prior to transplantation and that transplantation of MSCs into aged patients will be less efficacious.

Stem cell rescue and rejuvenation

Strategies allowing generation of large numbers of MSCs that have retained their stemness and rescue of senescent MSC in disease conditions are needed for clinical applications. Fig. 29.2 summarized current research efforts to prevent MSC senescence and achieve their rejuvenation, including genetic engineering, pharmacologic approaches, senolytics drugs, and immune-cell modulation. Genetic engineering targeting key pathways can slow MSC aging, such as SIRTs upregulation, p16, and/or p21 depletion. However, genetic manipulation runs the risk of tumorigenicity and genomic instability, which limit its human application. Pharmacologic approaches and cytokine supplementation have shown promise for delaying senescence and might be more clinically applicable [91]. Below we discuss a few examples of these approaches that have been applied in kidney diseases.

Given the pivotal role of p16 (INK4a) in senescence processing, a genetic engineering study [92] demonstrated that deletion of p16 (INK4a) ameliorated renal tubulointerstitial injury by inhibiting SASP in Bmi-1-deficient mice. However, whether p16 (INK4a) deletion is linked to MSC function is still unknown. Pharmacologic approaches to rescue MSC under senescent stress conditions have also been explored. Studies [93] showed protective effect of melatonin and pioglitazone on MSCs undergoing senescence induced by the uremic toxin, indoxyl sulfate, through regulation of the PPAR-γ, and cellular prion protein axis. Furthermore, Yun et al. [94] demonstrated that stem cell senescence in patients with CKD could be potentially rescued by administration of melatonin to restore the expression of senescence marker protein 30, which is normally suppressed because of the induction of the autophagy pathway in CKD patients by the uremic toxin p-cresol. Melatonin also showed therapeutic effects by successfully protecting CKD-MSCs from related ischemic conditions [95], thereby enhancing angiogenesis and survival. In a murine hindlimb ischemia model [96], melatonin-treated senescent MSCs enhanced functional

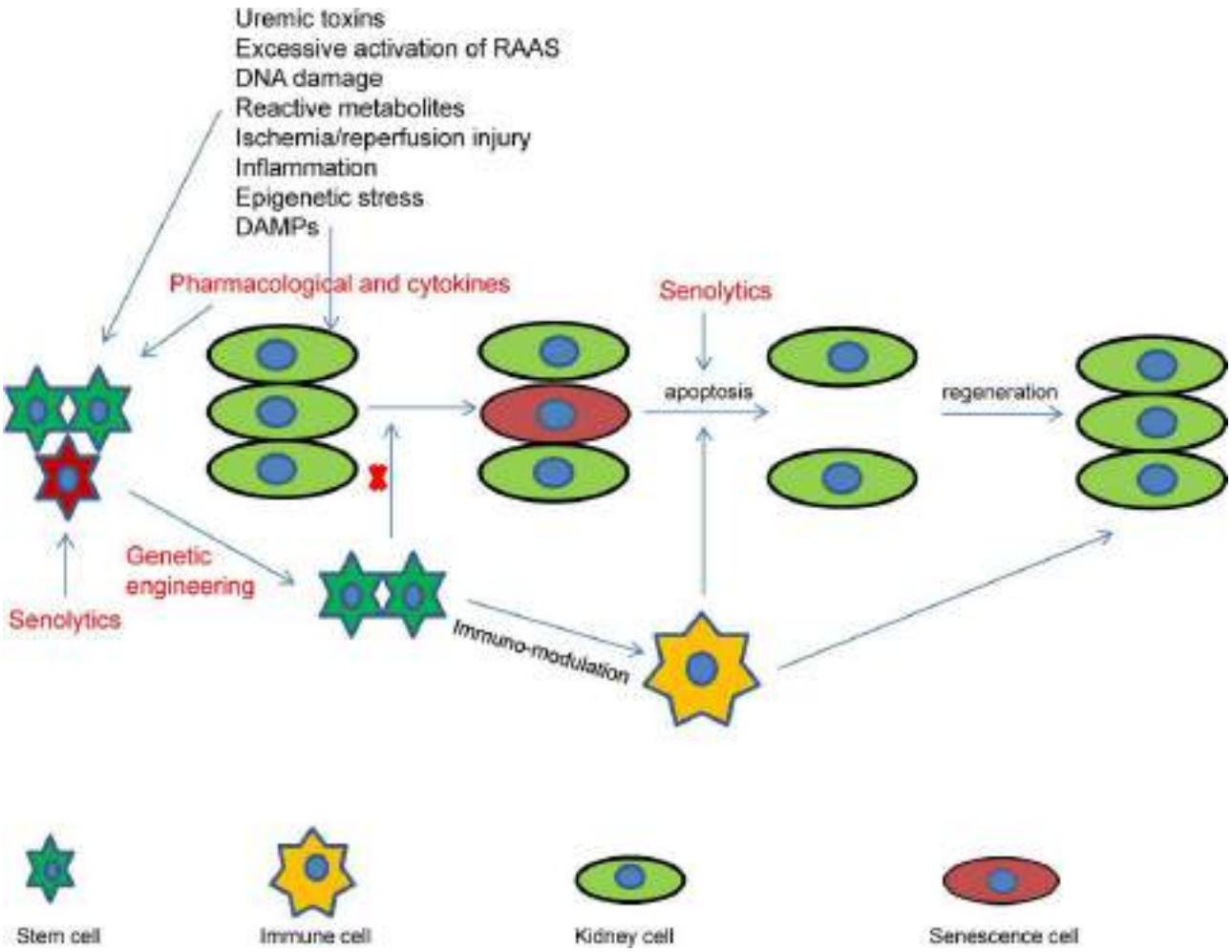

FIG. 29.2 Current efforts to prevent MSC senescence and induce rejuvenation, including genetic engineering, pharmacologic approaches and cytokine supplementation, senolytics drugs, and immune-cell modulation. *DAMPs*, damage-associated molecular pattern proteins; *RAAS*, renin angiotensin aldosterone system.

recovery by increasing blood flow perfusion, limb salvage, and neovascularization. Potentially, melatonin might be a clinically applicable strategy to protect MSCs from senescence during CKD.

Another study [97] showed that fucoidan, a marine sulfated polysaccharide, protects against p-cresol-induced cellular senescence in MSCs through activation of the FAK-Akt-TWIST pathway. Possibly, fucoidan could be used in conjunction with functional MSC-based therapies in the treatment of CKD.

Senolytic drugs that target senescent cells might thereby rejuvenate tissues. Four potentially senolytic drugs [98], navitoclax, quercetin, nicotinamide riboside, and danazol, have been tested in vitro on long-term expansion of MSCs with replicative senescence. Coculture experiments of fluorescently labeled early and late passages revealed that particularly navitoclax had a significant albeit moderate senolytic effect, reflected in reduced SA-beta-gal staining in senescent MSCs upon treatment with navitoclax. However, none of the drugs had significant effects on the maximum number of population doublings, telomere length, or epigenetic senescence predictions. It requires more investigation to identify potent senolytic drugs to meet the high hopes for regenerative medicine.

Senescent cells in tissues can activate both innate and adaptive immune responses to eliminate senescent cells and maintain tissue homeostasis. Natural killer (NK) cells, $CD4^+$ T-regulating cells (Treg), and macrophages are all involved in clearance of senescent cells. NK cells initiate a rapid, proinflammatory environment that is conducive to many forms of effective immune host defense. However, NK cells can also play protective role in allograft tolerance induction by regulating donor dendritic and Treg cells [99]. A study [100] showed that indoleamine 2, 3-dioxgenase-transfected MSCs increased the direct immunoregulatory properties of MSCs, enhanced the expression and function of CD4CD25 Foxp3 Treg cells, and induced allograft tolerance in kidney transplantation. A phase-I

clinical study [101] shows that expanded Tregs potently suppressed allogeneic responses and induced generation of new Tregs in the recipient's allo-responders in vitro, and all doses of Treg therapy tested were safe with no adverse effects. Whether Tregs infusion may induce transplant tolerance, thus avoiding long-term use of toxic immunosuppressive agents need more clinical trials. Recently, we found that MSC-derived extracellular vesicles can induce Treg cells to ameliorate chronic ischemic kidney injury [102], underscoring the important role of Treg cells in regenerative therapy for CKD. It is well known that MSC attenuates renal inflammation by promoting macrophages M1 to M2 polarization. However, the role of macrophages on MSC senescence, or vice versa, is unclear. Macrophage migration inhibitory factor (MIF), a soluble factor derived from activated T lymphocytes, inhibits random migration of macrophages. MIF also rescues MSCs from doxorubicin-induced senescence through the PI3K-Akt signaling pathway [103], and rejuvenates aged human MSC to improve myocardial repair [104]. Hence, MSC has close interactions with the immune system.

Thus, MSC and EPC may undergo senescence both in vivo during kidney diseases and in vitro at high passages. Although some potential rejuvenation strategies have been explored, the current knowledge of senescence and how to manipulate it is still limited. Given that functional attrition and reduced regenerative potential in stem cells are important aspects of aging in organisms, MSC rejuvenation holds considerable promise for broadening the applications of MSC.based therapy. Importantly, an increasing number of preclinical trials have reported therapeutic effects exerted by MSC-derived EVs via paracrine mechanisms in several disease models [105, 106]. Whether MSC-EVs are vulnerable to parent-cell senescence and how they can be identified are issues to be addressed in future studies.

In summary, senomorphics, senolytics, and other rejuvenation strategies show varying benefits in in vivo and in vitro models of kidney aging and disease. This variance is highly dependent on the cell type and underlying etiology of senescence. It is therefore important to investigate the senotherapeutic drugs in the cell type of interest using the appropriate and relevant stimuli of senescence. The senotherapies tested in kidney disease in vivo models show promising signs of kidney function preservation and reduced kidney fibrosis.

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CHAPTER

30

Natural products in regeneration

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Introduction

Renal damage can occur as a result of a variety of insults, such as nephrotoxins (e.g., cisplatin, heavy metals, aristolochic acid), chronic illness (e.g., diabetes, autoimmune diseases), and acute conditions (e.g., ischemia–reperfusion injury, rhabdomyolysis). During wound healing, macrophages infiltrate the injured tissue to clear damaged cells and regulate reparative responses, while fibroblasts deposit extracellular matrix to promote healing. However, these reparative processes are often dysregulated, especially during severe and chronic insults, resulting in tissue inflammation and fibrosis, and ultimately, long-term disease. Therefore, kidney inflammation and fibrosis present barriers to repair after renal injury. Regenerative capacity is also determined by nicotinamide adenine dinucleotide (NAD^+) production and consumption. In this chapter, we review natural products and their mechanisms of action that may provide benefit in renal regeneration, with specific focus on compounds that target inflammation and fibrosis, and that promote NAD^+ supply. Natural products that target inflammation and fibrosis are presented in two categories: (1) compounds that would be consumed in the diet or as a supplement and (2) compounds that are not likely to be consumed in the diet. These compounds, their chemical structures, plant sources, and reported renal effects are summarized in Table 30.1.

Natural products for renal regeneration

Dietary or supplemental natural products for renal inflammation and fibrosis

Non-flavonoid polyphenols: Curcumin, resveratrol

Curcumin is the primary biologically active substance in turmeric, a spice derived from the rhizomes of the plant *Curcuma longa* [107]. It binds directly to the proinflammatory molecules, TNFα, cyclooxygenase (COX)-1, and COX-2, and enhances NRF2 abundance, and thus is well known for its antiinflammatory activity [108]. Some human studies have reported benefits of turmeric supplementation on kidney function in patients with lupus nephritis [14] and with diabetic nephropathy [15]. In cadaveric kidney recipients, administration of a bioflavonoid preparation containing 480 mg curcumin and 20 mg quercetin (discussed below) once or twice daily for 1 month improved early kidney graft function [109]. Additionally, turmeric, curcumin alone, and a supplement containing *Curcuma longa* and *Boswellia serrata* have been shown to reduce plasma levels of proinflammatory cytokines in various disease states, including diabetic nephropathy, chronic kidney disease (CKD), and nonalcoholic fatty liver disease (NAFLD) [15, 110–113]. In rats with cisplatin-induced acute kidney injury (AKI), pretreatment with curcumin improved plasma

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TABLE 30.1 Natural products for renal regeneration.

Dietary and supplemental natural products				
Compound	**Chemical structure**	**Source**	**Reported renal effects**	**Disease context**
Curcumin	Polyphenol; curcuminoid	Turmeric (from *Curcuma longa*)	↑ NRF2 signaling [1] ↓ Proinflammatory signaling [2–5] ↓ EMT [1, 6, 7] ↓ TGF-β1 signaling [8] ↓ Fibrosis [3–5, 9–13]	AKI (cisplatin) [2] Lupus nephritis [14] Diabetic nephropathy [4, 5, 11, 12, 15] CKD [3, 9, 10] Obstructive nephropathy [13]
Resveratrol	Polyphenol; stilbene	Grapes, red wine, berries, peanuts	↑ NRF2 signaling [16, 17] ↓ Oxidative stress [16, 17] ↓ Macrophage TLR4-NFκB signaling [18] ↓ Inflammation [16, 19, 20] ↓ Fibroblast activation [17] ↓ Fibrosis [17]	AKI (sepsis) [18] Renal I/R injury [16] Diabetic nephropathy [17, 19] Polycystic kidney disease [20]
Epigallocatechin-3-gallate (EGCG)	Flavonoid; catechin	Green tea, black tea, white tea	↑ NRF2 signaling; ↓ oxidative stress [21–27] ↓ Proinflammatory cytokines [23–25] ↓ Macrophage infiltration [26, 27] ↓ NF-κB signaling [22, 27] ↓ EMT [28, 29] ↓ TGF-β1 signaling [28, 29] ↓ Fibrosis [21, 28, 29]	AKI (contrast agent, gentamicin, cisplatin) [23–25] Renal I/R injury [30] Diabetic nephropathy [21] Obstructive nephropathy [29, 31] Cadmium-induced nephrotoxicity [28] Lupus nephritis or glomerulonephritis [22, 26, 27]
Rutin	Flavonol	Citrus fruits, buckwheat, berries, capers	↓ Oxidative stress [32–36] ↑ Antioxidant response and NRF2 signaling [32–36] ↓ Proinflammatory signaling [32–38] ↓ NLRP3 inflammasome activation [39] ↓ EMT; ↓ TGF-β1 signaling; ↓ fibrosis [37, 38]	AKI (sepsis, $HgCl_2$, vancomycin, gentamicin, cisplatin) [32–36] Obstructive nephropathy [37] CKD [38] High fructose-induced hyperuricemia [39]
Troxerutin	Flavonol	*Sophora japonica* (i.e., Japanese pagoda tree), tea, coffee, cereal grains	↓ Oxidative stress [40–42] ↑ Antioxidant enzymes [41, 42] ↓ Proinflammatory signaling [40–43] ↓ CXCR4-TXNIP/NLRP3 signaling [40] ↓ TGF-β abundance [43]	Chronic renal injury (exogenous toxins) [40, 41, 43] Obstructive nephropathy [42]
Quercetin	Flavonol	Citrus fruits, apples, onions, grapes, broccoli	↓ Proinflammatory cytokines [44–46] ↓ TLR4-MyD88-NF-κB signaling [45, 47] ↓ NLRP3 inflammasome signaling [46] ↓ EMT [47, 48]; ↓ TGF-β1 signaling [45]; ↓ Fibroblast activation [47, 48] ↓ mTOR signaling [47, 49] ↓ β-Catenin signaling [47] ↓ Hedgehog signaling [48] ↓ Fibrosis [47–49]	AKI (cisplatin) [44] Obstructive nephropathy [45, 47, 48] Diabetic nephropathy [46]
Genistein	Isoflavone	Soybeans, fava beans, lupin, coffee	↑ NRF2 signaling and antioxidant enzymes [50] ↓ Oxidative stress [50] ↓ NF-κB signaling [51–53] ↓ EMT [54] ↓ TGF-β1 signaling [50] ↑ Klotho (antifibrotic) [54] ↓ Fibrosis [50, 54]	AKI (cisplatin) [51] Renal I/R injury [52] Aging [53] Obstructive nephropathy [54] Diabetic nephropathy [50]

TABLE 30.1 Natural products for renal regeneration—cont'd

Dietary and supplemental natural products				
Compound	**Chemical structure**	**Source**	**Reported renal effects**	**Disease context**
Berberine	Benzyl-isoquinolone alkaloid	Barberry, Oregon grape, goldenseal, tree turmeric	↓ Oxidative stress [55, 56] ↑ Mitochondrial biogenesis [55] ↓ TLR4-NF-κB signaling [56–59] ↓ Macrophage infiltration [56] ↓ TGF-β1 abundance [59–61] ↓ Fibrosis [55, 57, 59–61]	AKI (cisplatin) [62] Diabetic nephropathy [57–59] Obstructive nephropathy [56]
Non-dietary natural products				
Compound	**Chemical structure**	**Source**	**Reported renal effects**	**Disease context**
Tanshinone IIA	Phenanthrenequinone; diterpene	*Salvia miltiorrhiza* Bunge (i.e., red sage, Danshen), an herb	↑ NRF2 signaling [63] ↓ Oxidative stress [63] ↓ GSK3β activation [64] ↓ NF-κB signaling [65] ↓ TGF-β1 signaling [65, 66] ↓ Fibrosis [65, 66]	AKI (contrast agent, folic acid) [63, 64] Renal I/R injury [64] CKD [65, 66]
Dihydrotanshinone			↓ TLR4-MyD88-NF-κB signaling [67] ↓ Macrophage NO production and proinflammatory cytokine secretion [67]	Sepsis-induced AKI [67]
Cryptotanshinone			↑ NRF2 signaling [68] ↓ Oxidative stress [68] ↓ NF-κB activation [68] ↓ Macrophage infiltration [68] ↓ EMT [69] ↓ Fibrosis [68, 69]	Obstructive nephropathy [68, 69]
Salvianolic acid A	Polyphenol		↓ Oxidative stress ↑ Antioxidant enzymes [70, 71] ↑ NRF2 signaling [71, 72] ↑ AMPK signaling [72] ↑ Sirtuin 1 signaling [73] ↑ Autophagy [73] ↓ p38-NF-κB signaling [74] ↓ Fibrosis [72, 74]	AKI (sepsis) [75] Doxorubicin-induced nephropathy [70] CKD [71, 74] Diabetic nephropathy [72, 73]
Salvianolic acid B			↓ Oxidative stress [76, 77] ↑ NRF2 signaling [76] ↓ Proinflammatory cytokines [77] ↓ NF-κB abundance [77] ↑ Sirtuin 1 [136] ↑ Autophagy [78] ↓ EMT [78–81] ↓ Fibroblast activation [82, 83] ↓ Fibrosis [78, 84]	AKI (contrast agent) [76] Renal I/R injury [77] Mercuric chloride-induced nephropathy [84] CKD [78]
Baicalin	Flavone glycoside	*Scutellaria baicalensis* (i.e., Baikal skullcap), an herb	↑ NRF2 signaling [85] ↓ Oxidative stress [85, 86] ↓ TLR2/4-NF-κB signaling [86] ↓ Proinflammatory cytokines [85, 86] ↓ EMT [87] ↓ TGF-β1 signaling [87] ↓ Fibrosis [87–89] ↑ miR-124 (antifibrotic) [89]	AKI (cardiac surgery) [85] Renal I/R injury [86] Diabetic nephropathy [88, 89] Obstructive nephropathy [87]
Wogonin	*O*-methylated flavone		↓ Proinflammatory cytokines ↓ NF-κB signaling [90–92] ↓ RIPK1/3-mediated necroptosis [91] ↓ TGF-β1 signaling [93]	AKI (cisplatin) [90, 91] Cadmium-induced nephrotoxicity [92] TGF-β1-treated tubular epithelial cells [93]
Wogonoside	Flavone glycoside		↓ TGF-β1 signaling [93]	TGF-β1-treated tubular epithelial cells [93]

Continued

TABLE 30.1 Natural products for renal regeneration—cont'd

Non-dietary natural products				
Compound	**Chemical structure**	**Source**	**Reported renal effects**	**Disease context**
Ligustrazine	Alkylpyrazine	Chuan Xiong (from *Ligusticum wallichii*, an herb)	↓ Proinflammatory cytokines [94] ↓ NF-κB signaling [95] ↓ Macrophage infiltration [96] ↓ EMT ↓ Myofibroblast activation [96] ↓ Fibrosis [96]	Contrast agent-induced nephropathy [94] Diabetic nephropathy [97] Obstructive nephropathy [96] TGF-β1-treated tubular epithelial cells [96]
Oxymatrine	Quinolizidine alkaloid	*Sophora flavascens*, a shrub	↓ Proinflammatory cytokines [98, 99] ↓ NF-κB signaling [98] ↓ Myofibroblast activation [98] ↓ EMT [100] ↓ TGF-β1 signaling [98, 100] ↓ Fibrosis [98–100]	Diabetic nephropathy [99, 100] Obstructive nephropathy [98]
Ergone	Steroid	*Polyporus umbellatus*, a fungus	↑ NRF2 signaling [101] ↓ Macrophage infiltration [102] ↓ Proinflammatory signaling [101] ↓ EMT [101] ↓ Fibrosis [102, 103]	CKD [102, 103] Aristolochic acid-induced nephropathy [104] Angiotensin II-treated tubular epithelial cells [101]
Alisol B 23-acetate	Triterpene	Rhizoma Alismatis (i.e., rhizomes of water-plantain *Alisma plantago-aquatica*)	↑ NRF2 signaling [101] ↓ Proinflammatory signaling [101] ↓ EMT [101, 105] ↓ TGF-β1 signaling [105] ↓ Wnt/β-catenin signaling [101] ↓ RAAS activation [105]	CKD [105] Angiotensin II-treated tubular epithelial cells [101]
Poricoic acids	Tetracyclic triterpene	*Poria cocos*, a medicinal mushroom	↓ EMT; ↓ TGF-β1 signaling ↓ Wnt/β-catenin signaling ↓ RAAS activation ↓ Fibrosis [106]	Obstructive nephropathy Angiotensin II- and TGF-β1-treated tubular epithelial cells [106]

AKI, acute kidney injury; *CKD*, chronic kidney disease; *EMT*, epithelial-to-mesenchymal transition; *I/R*, ischemia–reperfusion; *RAAS*, renin-angiotensin-aldosterone system. Natural products that promote NAD^+ supply are not included in this table.

indicators of kidney function, reduced renal proinflammatory signaling, such as NF-κB abundance and expression of TNFα and IL-6, and upregulated expression of the antiinflammatory cytokine IL-10 [2]. The antiinflammatory effects of curcumin have also been shown in rat models of CKD [3] and diabetic nephropathy [4], and in mouse macrophages exposed to high glucose [5]. Curcumin may also impact fibrotic processes, as it has been shown to improve renal fibrosis in various rat models of CKD [3, 9, 10], rodent models of diabetic nephropathy [4, 5, 11, 12], and mice with obstructive nephropathy [13]. In a rat kidney tubular epithelial cell line exposed to high glucose, curcumin reduced epithelial-to-mesenchymal transition (EMT), which was partly dependent on its ability to induce NRF2 [1]. In immortalized human proximal tubular cells (HK − 2) treated with TGF-β1, curcumin was shown to suppress EMT [6, 7] and TGF-β1 signaling [8].

Resveratrol is a polyphenol belonging to the stilbene family and is common in human diets through consumption of grapes, red wine, berries, and peanuts [107]. It is most widely recognized for its ability to promote Sirtuin 1 activation and processes that contribute to longevity [114, 115], but also has diverse antiinflammatory and antioxidant effects, including inhibition of NF-κB activity and interleukins, and activation of NRF2 signaling [116]. While resveratrol has not been studied extensively in human kidney disease, some studies show that individuals with type 2 diabetes taking a daily resveratrol supplement (10–250 mg) for at least 1 month exhibit improvements in plasma or urine indicators of kidney function, and improvements in glucose homeostasis [117, 118]. Moreover, daily supplementation with resveratrol for at least 3 months has been found to reduce plasma levels of proinflammatory cytokines in patients with Alzheimer's disease [119], rheumatoid arthritis [120], and NAFLD [121]. In mice with sepsis-induced AKI, both pretreatment and intervention with resveratrol reduced renal inflammation, which was associated with reduced TLR4-NF-κB signaling in renal macrophages [18]. Pretreatment of rats with resveratrol prior to renal ischemia–reperfusion injury improved kidney function, reduced renal inflammation, and reduced oxidative stress likely through NRF2 induction [16]. The renal antiinflammatory effects of resveratrol have been further

demonstrated in rodent models of diabetic nephropathy and polycystic kidney disease [19, 20]. Like curcumin, resveratrol may also impact fibrotic processes. Resveratrol has been reported to attenuate fibrosis in mice with diabetic nephropathy [17]. Using high glucose conditions to promote rat renal fibroblast activation, He et al. demonstrated that resveratrol may mitigate renal fibrosis by reducing fibroblast activation in the kidney through activation of AMPK and reduction of NOX4-derived reactive oxygen species (ROS) [17].

Flavonoids: Epigallocatechin-3-gallate, rutin, quercetin, genistein

Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea, and while green tea is the primary source of EGCG in the diet, it can also be consumed in white or black tea [122]. EGCG is a potent ROS scavenger [123] and induces NRF2 [21, 22, 124, 125], but also has direct antiinflammatory actions, including inhibition of NF-κB activity and inhibition of proinflammatory cytokine and chemokine expression [126]. In patients with diabetic nephropathy, supplementation with green tea polyphenols (41% of supplement consisting of EGCG) normalized urinary albumin-to-creatinine ratio, indicative of improved kidney function, and reduced serum TNFα [127]. In an 8-week randomized, double-blind, split-face clinical trial, treatment of facial acne with topical 1% and 5% EGCG reduced acne and inflammatory lesions, and reduced expression of IL-8 and NF-κB in skin; similar antiinflammatory effects were observed in human sebocytes exposed to heat inactivated *Propionibacterium acnes* [128]. In rodents with AKI induced by cisplatin, gentamicin or the contrast agent iopromide, treatment with EGCG normalized plasma creatinine and blood urea nitrogen (BUN), reduced renal oxidative stress, and reduced renal levels of proinflammatory cytokines [23–25]; in mice with contrast agent-induced nephropathy, EGCG also upregulated NRF2 and its target heme oxygenase-1 (HO-1) [25]. Treatment of mice with EGCG prior to renal ischemia–reperfusion injury improved plasma indices of kidney function, reduced renal inflammatory markers, and reduced renal fibrosis [30]. EGCG has also been found to provide benefit in rodent models of chronic nephropathy, such as an chronic cadmium exposure [28] and obstructive nephropathy [29, 31], in which it reduces renal inflammation [29, 31] and fibrosis, as well as profibrotic processes such as EMT and TGF-β1 signaling [28, 29]. Moreover, in mouse models of immune-mediated glomerulonephritis and lupus nephritis, pretreatment and/or intervention with EGCG improved renal function, reduced kidney macrophage and lymphocyte infiltration [26, 27], reduced renal NF-κB activation [22, 27], and reduced oxidative stress and upregulated NRF2 [22, 26, 27]. Peng et al. showed that the beneficial effects of EGCG on kidney function may be PPARγ-dependent [27], while Sun et al. used genetic deletion of *Nrf2* to show that EGCG improves renal fibrosis and oxidative stress in mice with diabetic nephropathy in an NRF2-dependent manner [21]. These antiinflammatory and antifibrotic effects of EGCG have also been shown in kidney epithelial cells and mesangial cells in vitro [129–132].

Rutin, also known as rutoside, quercetin-3-rutinoside, and sophorin, is a citrus flavonol and potent antioxidant that can also be extracted from buckwheat [133], berries [134], and capers [135], among other food plants. Although there are have not been clinical studies performed to investigate the specific effects of rutin on kidney function, one study showed that female day-care staff that consumed rutin-rich tartary buckwheat cookies exhibited a decrease in serum myeloperoxidase, indicative of reduced systemic inflammation, which was not observed in those who consumed common buckwheat cookies [136]. In rodent models of acute nephrotoxicity induced by sepsis [32], mercuric chloride exposure [33], and administration of vancomycin [34], gentamicin [35], and cisplatin [36], pretreatment and/or coadministration with rutin improved plasma indices of renal function, improved renal antioxidant responses, reduced lipid peroxidation and oxidative stress in the kidney, and reduced renal expression of proinflammatory mediators, including NF-κB, TNFα, and IL-1β [32–36]. Rutin has also been shown to reduce renal inflammation and fibrosis, TGF-β1 signaling, and markers of EMT in rat models of obstructive nephropathy [37] and CKD [38], and to reduce renal NRLP3 inflammasome activation in rats with fructose feeding-induced hyperuricemia and hyperlipidemia [39].

Quercetin is a flavonol found at high concentrations in onions, apples, grapes, wine, broccoli, citrus fruits, and tea [137], and has potent antioxidant [138] and antiinflammatory [139] properties. In patients with chronic diseases such as rheumatoid arthritis [140], cardiovascular disease [141], and obesity [142], supplementation with quercetin has been shown to reduce circulating levels of TNFα and improve other metrics of disease severity [140–142]. However, its antiinflammatory effects appear to be context-dependent, as quercetin supplementation does not reduce systemic inflammation after exercise [143] or in healthy individuals [144]. In mice with cisplatin-induced AKI, intervention with quercetin improved plasma indices of kidney function, reduced renal expression of proinflammatory cytokines and NF-κB signaling, and promoted M2 macrophage polarization and inhibited M1 macrophage polarization. These antiinflammatory effects may be dependent on the ability of quercetin to inhibit Mincle, a pattern recognition receptor involved in the immune response [44]. In rodent models of obstructive nephropathy, pretreatment or intervention with quercetin appears to reduce renal inflammation, indicated by decreased TLR4-MyD88-NF-κB signaling [45, 47],

and to limit fibrosis, indicated by reduced TGF-β1 signaling [45], reduced hedgehog signaling [48], inhibition of EMT [47, 48], and reduced fibroblast activation [47, 48]. Quercetin has been further observed to reduce renal inflammation and specifically NLRP3 inflammasome signaling in rats with diabetic nephropathy [46]. A 2015 study conducted by Lu et al. reported that, in streptozotocin-induced diabetic rats, treatment with quercetin daily for 2 weeks reduced renal fibrosis and inhibited EMT, possibly through inhibition of mTOR signaling, as the specific mTOR inhibitor rapamycin elicited similar antifibrotic effects in this model [49]. This inhibitory effect of quercetin on mTOR signaling has also been observed in mice with obstructive nephropathy and in TGF-β1-stimulated rat kidney fibroblasts [47].

Genistein is an isoflavone enriched in soybeans [145], fava beans, lupin, [146] and coffee [147], with antiinflammatory properties, including inhibition of NF-κB activity and proinflammatory cytokine secretion [148] and antioxidant effects [50, 149]. Eight weeks of genistein supplementation has been shown to reduce serum levels of IL-6 and TNFα, and to elicit other metabolic improvements in NAFLD patients [150]. Soy supplementation has been shown to reduce leukotriene C_4 synthesis in eosinophils from asthma patients [151] and may reduce plasma CRP in end-stage renal disease [152]. In mice with cisplatin-induced AKI, treatment with genistein for 3 days improved kidney function and reduced renal immunohistochemical staining for MCP1, F4/80, and NF-κB [51]. Genistein has also been shown to reduce renal TLR4 and TNFα mRNA in rats with renal ischemia–reperfusion injury [52] and to reduce NF-κB activation in aged rat kidneys and in angiotensin II-treated rat endothelial cells [53]. Antifibrotic effects of genistein have been reported in rodent models of chronic renal injury, such as obstructive nephropathy and diabetic nephropathy [50, 54]. Li et al. showed that, in a mouse model of obstructive nephropathy, pretreatment with genistein dramatically reduced kidney fibrosis and inhibited renal EMT, and further showed that these effects were dependent on the kidney-enriched antiaging and antifibrotic protein Klotho [54].

Berberine

Berberine is an alkaloid found in the roots and rhizomes of various plants including, but not limited to, barberry, Oregon grape, goldenseal, and tree turmeric, but is most often consumed as a supplement. Berberine is well known for its beneficial effects in metabolic disease, such as its ability to lower serum lipids [153–157] and blood glucose [155, 156, 158], and has also been shown to provide benefit in NAFLD [159]. While the clinical evidence supporting its potential antiinflammatory effects is more limited, some studies suggest that berberine can reduce plasma CRP, IL-6, and MCP-1 in acute coronary syndrome patients receiving percutaneous coronary intervention [157, 160]. In rodent models of diabetic nephropathy, treatment with berberine has been shown to improve glucose homeostasis and kidney function, reduce renal inflammation, indicated by reduced renal TLR4-NF-κB signaling [57–59], and reduce renal fibrosis, indicated by reduced collagen deposition and reduced expression of several fibrotic markers [55, 57, 59–61]. Berberine has also been observed to reduce renal inflammation in mice with cisplatin-induced AKI [62] and to reduce renal inflammation and fibrosis in rats with obstructive nephropathy [56]. Furthermore, the antiinflammatory effects of berberine have been shown in THP-1 macrophages exposed to oxidized LDL [157], and in mouse podocytes exposed to excess glucose [161].

Nondietary natural products for renal inflammation and fibrosis

Compounds from roots of Salvia miltiorrhiza: Tanshinones and Salvianolic acids

Salvia miltiorrhiza Bunge, also known as red sage or Danshen, is a traditional Chinese medicine [162] used in the treatment of several chronic diseases, including cardiovascular diseases, liver cirrhosis, and chronic renal failure, among others [162]. **Tanshinones**, including **tanshinone IIA**, **dihydrotanshinone**, and **cryptotanshinone**, are lipophilic phenanthrene compounds extracted from the roots of *S. miltiorrhiza* and have various antioxidant and antiinflammatory properties [163–165]. While there have been few clinical trials studying tanshinones, coronary artery disease patients treated with sodium **tanshinone IIA** sulfate in combination with statin therapy exhibited reduced plasma levels of several inflammatory mediators including CRP, IL-6, MCP1, and sCD40L compared to patients receiving statin therapy alone [166]. Moreover, two recent meta-analyses reported renal benefits of sodium tanshinone IIA sulfonate in patients with hypertensive nephropathy [167] and in CKD patients [168]. In rats with contrast agent-induced nephropathy, pretreatment with tanshinone IIA improved kidney function and histopathology, reduced renal apoptosis and oxidative stress, and activated renal NRF2 signaling [63]. Additionally, in mice with AKI induced by folic acid administration or renal ischemia–reperfusion injury, intervention with tanshinone IIA reduced renal inflammation and fibrosis by blunting GSK3β activation [64]. The antiinflammatory and antifibrotic effects of tanshinone IIA have been further demonstrated in rat models of CKD [65, 66], in which it reduces TGF-β1-Smad

signaling [65, 66] and NF-κB signaling [65]. A 2019 study by Yuan et al. reported that **dihydrotanshinone** improved kidney function and systemic inflammation in mice with sepsis-induced AKI, and reduced TLR4-MyD88-NF-κB signaling, nitric oxide (NO) production, COX-2 and iNOS abundance, and proinflammatory cytokine secretion in LPS-treated macrophages [67]. In mouse models of obstructive nephropathy, intervention with **cryptotanshinone** has been shown to reduce NF-κB activation and macrophage infiltration into kidneys [68], upregulate antioxidant defenses and reduce oxidative stress [68], reduce renal fibrosis [68, 69], and reduce renal EMT [69]. An interesting study conducted by Gao et al. showed that a total tanshinone preparation had potent antiinflammatory effects in mice with sepsis-induced AKI and in LPS-treated macrophages, and that total tanshinones elicited a greater decrease in macrophage NO production than any individual tanshinone alone [169].

Salvianolic acids are polyphenols extracted from the roots of Danshen that have potent antioxidant properties [170, 171]. There is some evidence that **salvianolic acid B**, which is more abundant in Danshen than salvianolic acid A, may reduce liver fibrosis in patients with chronic hepatitis C [172], but this has yet to be reproduced by other groups. The antifibrotic effect of salvianolic acid B in liver is likely due to its ability to inhibit hepatic stellate cell activation [173]. In rats with contrast agent-induced AKI, pretreatment with salvianolic acid B improved kidney function and histopathology and reduced renal oxidative stress likely through activation of PI3K-Akt-NRF2 signaling [76]. Moreover, in rats with renal ischemia–reperfusion injury, pretreatment with salvianolic acid B upregulated antioxidant responses, reduced oxidative stress, and reduced renal expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α and of NF-κB p65 [77]. Salvianolic acid B has also been shown to reduce renal fibrosis in mice administered mercuric chloride [84] and in a rat model of CKD [78]. The latter study also showed that salvianolic acid B reduced EMT and activated autophagy both in rats with CKD and in TGF-β1-treated HK-2 cells in a Sirtuin 1-dependent manner [78]. This ability of salvianolic acid B to inhibit EMT has been reproduced by other groups [79–81], and may also involve upregulation of the miR-106b-25 cluster, which has been shown to target the TGF-β type II receptor [81]. Salvianolic acid B has also been shown to reduce TGF-β1-induced kidney fibroblast activation [82] and high glucose-induced proliferation and fibronectin secretion from mesangial cells [83]. There is some evidence from studies in rats with doxorubicin-induced nephropathy [70] and mice with sepsis-induced AKI [75] suggesting that **salvianolic acid A** can improve kidney function, reduce renal inflammation [70, 75], and reduce renal oxidant stress [70]. Furthermore, in rat models of CKD, treatment with salvianolic acid A has been shown to reduce renal expression of proinflammatory cytokines and chemokines, renal MAPK p38-NF-κB signaling, and tubulointerstitial fibrosis [74], and to reduce renal oxidative stress by activation of NRF2 signaling [71]. Wu et al. reported that diabetic mice administered salvianolic acid A in combination with metformin showed greater improvements in kidney function, renal inflammation, and renal fibrosis compared to mice treated with either compound individually, possibly resulting from concurrent activation of NRF2 (salvianolic acid A) and AMPK (metformin) [72]. Moreover, in rats with diabetic nephropathy, treatment with salvianolic acid A improved fasting blood glucose and serum lipids, reduced renal inflammation and oxidative stress, and restored renal Sirtuin 1 signaling and autophagy [73].

Flavonoids from roots of Scutellaria baicalensis: Baicalin, wogonin, wogonoside

Baicalin is a flavone glycoside isolated from the roots of the herb *Scutellaria baicalensis*, also known as Baikal skullcap, which is used in traditional Chinese medicine. In patients with both rheumatoid arthritis and coronary artery disease, supplementation with baicalin for 12 weeks improved plasma lipid profiles, reduced plasma CRP and cardiotrophin-1, and improved rheumatoid arthritis symptoms, with the latter two effects indicative of reduced inflammation [174]. There is also some weak evidence suggesting that baicalin may improve kidney function in pediatric sepsis-induced AKI patients [175]. In rats with AKI resulting from cardiac surgery [85] or renal ischemia–reperfusion injury [86], pretreatment with baicalin improved kidney function, reduced renal oxidative stress [85, 86] and upregulated NRF2 signaling [85], and reduced renal expression of proinflammatory cytokines [85, 86] and TLR2/4-NF-κB signaling [86]. Antiinflammatory effects of baicalin have been shown in HK-2 cells cultured in a diabetic milieu consisting of high glucose, advanced glycation end products, and TNFα, in which it reduces NF-κB and JAK–STAT signaling [176]. Baicalin has also been shown to reduce renal fibrosis in rodent models of obstructive nephropathy [87] and diabetic nephropathy [88, 89]. Specifically, baicalin appears to inhibit EMT and TGF-β1 signaling in rats with obstructive nephropathy and in TGF-β1-treated rat epithelial cells [87]. The antifibrotic effects of baicalin may also be related to its ability to induce miR-124, which reduces renal fibrosis in diabetic nephropathy and targets the TLR4-NF-κB signaling pathway [89].

Similar to baicalin, **wogonin** is an *O*-methylated flavone extracted from *S. baicalensis* with antioxidant and antiinflammatory properties [177, 178]. Although the clinical evidence in support of the antiinflammatory effects of wogonin is limited, this flavonoid has been shown to induce apoptosis in human neutrophils [179].

Furthermore, in rodents with cisplatin-induced AKI, pretreatment [90] or intervention [91] with wogonin improved kidney function and reduced renal inflammation, as indicated by reduced expression of proinflammatory cytokines, reduced macrophage infiltration, and reduced NF-κB signaling. Meng et al. reported that wogonin may elicit antiinflammatory effects in cisplatin-induced AKI by inhibiting RIPK1/3-mediated necroptosis [91]. Antiinflammatory and antioxidant effects of wogonin in the kidney have also been demonstrated in rats with cadmium-induced nephrotoxicity [92]. Although the potential antifibrotic effects of wogonin in the kidney have not been studied in vivo, wogonin has been shown to inhibit TGF-β1-induced production of collagen I and α-smooth muscle actin in human and mouse tubular epithelial cells, likely through inhibition of Smad3 phosphorylation [93]. In the same study, it was observed that **wogonoside**, another flavonoid derived from *S. baicalensis*, also reduced TGF-β1-induced fibrotic responses in tubular epithelial cells, albeit with less potency than wogonin [93].

Ligustrazine

Ligustrazine, also known as tetramethylpyrazine, is an alkylpyrazine extracted from Chuan Xiong, a dry rhizome of the Chinese herb *Ligusticum wallichii*, that is widely used in China for the treatment of vascular diseases [180]. Ligustrazine has been shown to provide benefit in patients with bronchial asthma [181] and to promote healing in patients with pressure sores [182]. Additionally, when combined with standard therapy, ligustrazine was shown to reduce the incidence of contrast agent-induced nephropathy in unstable angina patients undergoing coronary angiography and/or percutaneous coronary intervention [183]. Notably, a meta-analysis of 25 randomized controlled trials conducted by Wang et al. suggests that ligustrazine improves renal function in patients with diabetic nephropathy [97]. Studies in rodents and cultured cells indicate that this clinical benefit may be related to its antiinflammatory properties. In rats with contrast agent-induced nephropathy, pretreatment with ligustrazine improved kidney function, normalized renal mitochondrial dynamics, and reduced renal expression of CCL2, CCR2, IL-6, and TNFα [94]. Similar antiinflammatory effects were observed in rat tubular epithelial cells treated with gentamicin, in which ligustrazine reduced NF-κB DNA binding and TNFα secretion [95]. Ligustrazine has also been shown to reduce renal macrophage infiltration and fibrosis in a rat model of obstructive nephropathy [96]. The antifibrotic effects of ligustrazine have been further shown in TGF-β1-treated HK-2 cells, in which it reduces EMT and myofibroblast activation [96].

Oxymatrine

Oxymatrine is a quinolizidine alkaloid extracted from the root of the Chinese herb *Sophora flavescens* with antioxidant, antiinflammatory, and antifibrotic properties [184–186]. Treatment with oxymatrine has been shown to improve liver function in chronic hepatitis B patients [187] and may provide benefit for liver fibrosis downstream of chronic viral hepatitis [188]. In patients with severe plaque psoriasis, both oxymatrine and the conventional therapy were shown to reduce disease severity, but only oxymatrine reduced the frequency of relapse after treatment withdrawal [189]. In mice with obstructive nephropathy, intervention with oxymatrine for 6 days reduced renal fibrosis and myofibroblast activation, and reduced renal expression of proinflammatory cytokines; these effects occurred in association with reduced TGF-β1 and NF-κB signaling [98]. In further support of its antiinflammatory and antifibrotic effects, rats with diabetic nephropathy administered oxymatrine orally for 11 weeks exhibit improved kidney function, as well as reduced renal expression of TNFα, TGF-β1, and CTGF [99]. The antifibrotic effects of oxymatrine may be mediated in part by inhibition of EMT and inhibition of TGF-β1 signaling [100].

Natural compounds that promote NAD^+ supply

NAD^+ production and requirements for cell and tissue health

NAD^+ is a highly conserved naturally occurring water-soluble cofactor that is vital to cellular metabolism. It stores energy in its reduced form (NADH), which can power ATP production. More recently, it has emerged that NAD^+ also serves as an essential co-enzyme for a host of indispensable functions within the cell. These functions include DNA repair, chromatin silencing, transcriptional regulation, metabolic switching, calcium mobilization, and lifespan regulation [190–193]. Rather than being based on redox events, these actions are based on NAD^+ consumption. Currently recognized mammalian NAD^+-consuming enzymes include: (1) ADP-ribose transferases and poly(ADP-ribose) polymerases (PARPs), (2) cADP-ribose synthases; and (3) Sirtuins. The diversity of functions requiring NAD^+ and its consumption is illustrated by the Sirtuin family of enzymes, which operate in the nucleus (Sirtuins 1, 2, 6, 7), cytosol (Sirtuins 1, 2), and the mitochondria (Sirtuins 3, 4, 5).

It is thus not surprising that there is complexity and redundancy in the biosynthetic pathways for NAD^+. Three main routes are recognized. NAD^+ is produced in a de novo pathway that begins with the essential amino acid L-tryptophan or aspartic acid. A second route entails the Preiss-Handler pathway, which converts nicotinic acid (also known as niacin or Vitamin B_3) into NAD^+ [190, 194]. A third critical pathway is the salvage pathway. Here, the intracellular generation of nicotinamide (NAM) begins an NAD^+ regeneration cycle by serving as a substrate for nicotinamide phosphoribosyl transferase (NAMPT). This produces nicotinamide mono-nucleotide (NMN), an immediate NAD^+ precursor. Importantly, naturally occurring nicotinamide riboside (NR) can also feed this cycle, through the nicotinamide riboside kinase-mediated conversion to NMN [190, 195, 196].

Despite the multiplicity of biosynthetic routes to NAD^+, there can be vulnerability in cellular NAD^+ content, particularly with aging and disease. Low NAD^+ levels will compromise NAD^+'s role in redox reactions and the host of NAD^+-consuming reactions that regulate cellular fitness and survival. In this context, NAD^+ "boosting," through supplementation with precursors such as nicotinic acid, NAM, NMN, and NR, may be an important determinant of health and disease. The benefits of specific NAD^+-boosting strategies could be tissue-dependent, given that the distribution of biosynthetic enzymes responsible for NAD^+ production varies among tissues. It is thus noteworthy that an enzyme that shunts precursors away from the de novo NAD^+ pathway, α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD), is predominantly found in the liver and kidney [197]. This could make the kidney a particularly important tissue for exogenously supporting the natural baseline NAD^+ stores.

NAD^+ effects on renal inflammation and fibrosis

Both acute and chronic NAD^+ supplementation strategies have been shown to be beneficial in animal models of renal disease, afforded by delivery of NR, NAM, or NMN [194]. In an inflammatory mouse model of ischemia–reperfusion injury, Tran et al. found that exogenous NAM supplementation protected renal function and aided in acute kidney recovery [198]. This was proposed to be related to promoting a PGC1α-dependent stress response. In addition, NMN has been reported to protect against both age-associated renal dysfunction and cisplatin-induced AKI [199]. This reno-protective effect was shown to be dependent on Sirtuin 1 activity in the kidneys. Using the same model of cisplatin-induced AKI, Giroud-Gerbetant et al. demonstrated that oral delivery of a reduced form of NR, NRH, markedly increased levels of NAD^+, beyond that of NR administration, and reduced renal injury compared to controls [200]. The authors related this to replenishment of NAD^+ levels, which are depleted in a PARP-dependent manner during cisplatin-induced injury. Zheng et al. also recently reported that NAM delivery reduced renal inflammation, as measured by reduction in inflammatory cytokines as well as cellular infiltration, in a unilateral urethral obstruction model of chronic renal interstitial fibrosis in mice. Moreover, TGF-β-induced renal fibrosis was inhibited in these mice, in an NAM dose-dependent manner [201].

There are also encouraging clinical data on NAD^+ supplementation strategies in humans [194]. Poyan Mehr et al. reported a beneficial response to NAM supplementation in patients undergoing elective, on-pump cardiac surgery [202]. Here, NAM was given at a dosage of up to 3 g once daily. The authors found good tolerability and a trend toward a reduced incidence of AKI. This phase I patient study was limited by the lack of significant renal impairment in the study population and overall was underpowered to observe any demonstrable clinical effects. In another phase I/II study published recently by Simic et al. a new compound called NRPT that combines NR and pterostilbene (PT), a Sirtuin activator found in blueberries, was found to be well tolerated in hospitalized patients with AKI [203]. The dose-escalation strategy revealed that therapy was safe, with no observed serious adverse events, and associated with a trend toward increased NAD^+ levels and improved renal function, compared to placebo controls.

NAD^+ effects on vascular inflammation and fibrosis

Kidney function is intimately related to vascular function, and diseases in both large and small blood vessels are central to renal disease. Accordingly, any impact of NAD^+ supplementation strategies on the vasculature is important when considering renal health.

Recently, the role of the rate-limiting enzyme in the NAD^+ salvage pathway, NAMPT, has been studied in aortic disease. Specifically, a smooth muscle cell-specific deficiency in NAMPT in mice promoted the formation of thoracic aortic aneurysms and worsened susceptibility to aortic wall dissection when stressed with angiotensin II [195]. Interestingly, supplementation with NR in NAMPT knockout mice protected vascular smooth muscle cells from oxidized DNA damage and significantly improved cell survival to near baseline. There has also been recent support for niacin supplementation in diminishing angiotensin II and calcium chloride-induced aortic adventitial inflammation and abdominal aneurysm formation in mice [204]. In this case, the beneficial effects of nicotinamide were shown to be related to Sirtuin 1 activity.

In another study, aortic inflammation in older mice, as indicated by NF-κB levels, was reversed by NMN supplementation [205]. This change was associated with an increase in Sirtuin 1 activity. Interestingly, other age-associated changes to the mouse aorta such as reduction in NO-dependent dilation, increased stiffness with decreased elastin, and increased collagen content were also reversed by NMN supplementation in old mice, to a level comparable to that of young mice.

The importance of augmenting NAD^+ supply to optimize endothelial cell lifespan, senescence, and angiogenic capacity has also been established [206, 207]. Furthermore, delivery of NAD^+ precursors has been shown to improve vascular endothelial dysfunction associated with age and inflammation [208, 209]. Das et al. found that NMN given to aged mice increased capillary density in skeletal muscle and exercise capacity [208]. This was shown to occur by Sirtuin 1-dependent pathways in the endothelial cells with enhanced exercise endurance, and was further augmented by hydrogen sulfide. The NAD^+ intervention was shown to sensitize endothelial cells to VEGF by suppressing Notch. Moreover, Hughes-Large et al. showed that endothelial dysfunction caused by palmitate lipotoxicity can be mitigated by niacin or NMN supplementation [209]. This functional rescue was shown to occur via niacin receptor activation.

As noted, NAD^+ supply pathways in vascular smooth muscle cells are also critical for lifespan, resistance to oxidative stress, and motility [210–213]. Li et al. showed that administration of NR partially reversed angiotensin II-induced phenotypic changes in vascular smooth muscle cells of the brain, as measured by loss of α-actin [214]. This protective effect of NR was also associated with reduced myelin degradation, improved tight junctions of the blood brain barrier, and reduced microglia activation along with functional improvements in memory.

Finally, there are emerging data on the impact of NAD^+-boosting strategies on the human vasculature. A human placebo-controlled clinical trial performed by Martens et al. in 2018 showed that NR supplementation up to 500 mg twice daily for 6 weeks was well tolerated and appeared to improve systolic blood pressure and reduced carotid artery stiffness [215]. This phase I study was performed on 30 healthy middle-aged individuals (age 55 to 79 years) and was underpowered to reliably detect clinical effects. Nevertheless, NAD^+ and even ATP levels were shown to be increased in patients who received therapy. Given the good tolerability of NAD^+ supplementation strategies in multiple studies, further investigations in this exciting area are warranted.

In summary, there are several natural compounds that promote NAD^+ production and more are in development. Attention to NAD^+ maintenance and supplementation is an evolving field of great interest with potential for significant translational impact on patient health.

Conclusion

In this chapter, we have reviewed natural compounds that may support renal regeneration, with specific focus on those with potential to mitigate renal inflammation and fibrosis, and to promote NAD^+ generation. Many of the compounds discussed have common underlying antiinflammatory and antifibrotic mechanisms, such as activation of NRF2 signaling, reduction of NF-κB signaling and proinflammatory cytokines, and reduction of EMT and TGF-β1 signaling (Fig. 30.1). There are some considerations for the use of natural products in chronic disease management. Many have poor oral bioavailability; to address this, there have been efforts to develop delivery methods and formulations to enhance absorption or minimize metabolism. It is also important to note that some compounds can elicit adverse effects at high doses and/or have been shown to inhibit drug metabolizing enzymes or drug transporters, and therefore have the potential to affect drug disposition. However, despite these considerations, many of these compounds show promise in both preclinical and clinical studies. Thus, continued efforts to enhance bioavailability and ensure safety of these natural products are warranted, as they have considerable potential to promote healthy renal regeneration and mitigate progression to long-term kidney disease.

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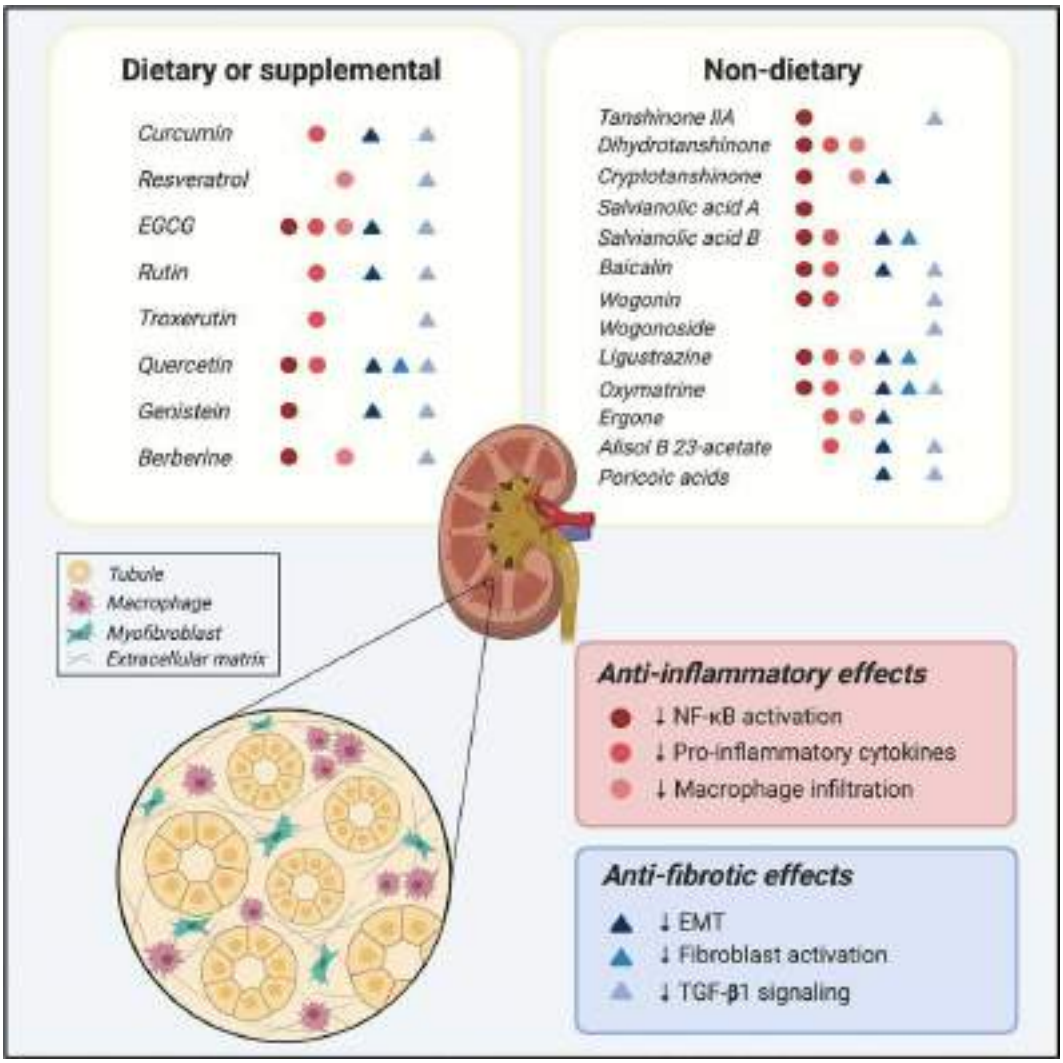

FIG. 30.1 Antiinflammatory and antifibrotic effects of natural compounds in the kidney. Natural compounds described in Table 30.1 are summarized above, with specific emphasis on renal antiinflammatory and antifibrotic effects that are common to many of the compounds. This figure is not an exhaustive summary of compound effects; for a more comprehensive summary see Table 30.1. Antiinflammatory effects are indicated by red-toned circles, and antifibrotic effects are indicated by blue-toned triangles. *EMT*, epithelial-to-mesenchymal transition; *EGCG*, epigallocatechin-3-gallate.

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CHAPTER

31

Nanotargeting to the kidney

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Nanoparticles in medicine

Interest in the application of nanotechnology to problems in nephrology has increased in recent years. Nanoscience is loosely defined as the investigation of materials where at least one dimension (either external or internal) is less than 100 nm but may be considered up to 1000 nm (1 μm) in some cases [1]. These materials may be composed of organic, inorganic, or biological components. The use of nanoparticles in medicine (a field sometimes called nanomedicine) most often includes applications related to drug delivery, imaging/diagnostics, and other therapeutic modalities, such as radiotherapy and photodynamic therapy.

Drug delivery strategies incorporating nanotechnologies have been in the clinic for 25 years and are designed to improve the pharmacologic properties of therapies. Improvements may include drug solubility, bioavailability, pharmacokinetics, biodistribution, the reduction of toxicities (especially dose-limiting toxicities), and drug resistance [2]. In addition, nanotechnologies are increasingly used, in several disease fields, to facilitate the administration of new types of drugs that may not be stable on their own in the body, such as RNA and protein therapies [3]. Nanoscientists have developed technologies to improve pharmacokinetic issues of many therapeutic drugs via encapsulation/protection of active cargos. Nanomaterials are also often developed to target specific tissues and control the release of drugs within those tissues [4].

Over the past several decades, nanoscale systems have been developed to modulate drug pharmacologic properties for cancer indications. The first clinically approved nanoparticle drug formulation, Doxil, incorporates the chemotherapeutic drug doxorubicin within lipid nanoparticles (LNPs) [5]. Doxil was developed to mitigate the cardiotoxicity of doxorubicin, which often causes cardiomyopathy. Doxil was approved by the FDA in 1995; since then, multiple nanoparticle formulations of anticancer drugs have reached the market.

Nucleic acids and gene editing technologies are subjects of significant excitement for their therapeutic potential [6]. The delivery of macromolecules such as proteins and oligonucleotides to their intended targets has been a challenge, in part due to the minimal stability of these large, fragile, molecules in the body [7]. Unmodified nucleic acids can also be cleaved by endonucleases in the serum and can activate innate immunity. Nucleic acids must reach the cytoplasm or nucleus of cells to find their pharmacologic targets. Transport of macromolecules to both the target cells and the target compartments of these cells presents a significant challenge [4, 8]. Nanoformulations of siRNA and mRNA with lipids and polymers have demonstrated the ability to abrogate siRNA degradation and facilitate in vitro or in vivo use [9]. Accordingly, the first FDA-approved small interfering RNA (siRNA) therapy, which targets the expression of the protein transthyretin in the liver to treat hereditary transthyretin amyloidosis, is a nanoparticle formulation [10].

Nanotechnology-based imaging agents have been developed for preclinical and clinical applications. Nanoparticles have been synthesized that incorporate radioisotopes to image tumors [11]. For instance, technetium sulfur colloid Tc-99m is a gamma ray-emitting particle used to measure several conditions including breast cancer metastases to the lymph nodes. It is often imaged in humans via single photon emission computer tomography (SPECT) [12, 13]. Ferumoxytol, an iron oxide nanoparticle, is used clinically for iron replacement therapy to treat anemia, but it has

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also been used in tumor imaging studies. It was employed in a clinical trial to assess prediction of tumor accumulation/efficacy of an irinotecan-encapsulated LNP [14].

Nanotechnologies have been developed for other therapeutic uses, such as radiotherapy and phototherapy. For example, a crystalline hafnium oxide nanoparticle was developed to synergize with radiotherapy. The interaction between ionizing radiation and hafnium was found to improve radiation-mediated cell death effected by radiotherapy procedures, discussed further below [15, 16].

In this chapter, we will discuss the current status of nanoparticle-based strategies to improve upon standard therapies, especially with respect to kidney disease. We will also detail existing nanomedicines in the clinic.

Nanoparticle pharmacokinetics

Nanoparticle targeting to specific organs in patients and animals can both increase the amount of drug at the target site, improve efficacy, and abrogate toxicities associated with systemic administration. Nanoparticle-based delivery can reduce chemotherapeutic toxicities by preventing exposure to certain sensitive tissues [17]. Even molecularly-targeted small molecules, such as kinase inhibitors, exhibit suppressed toxicity profiles when encapsulated in targeted nanoparticles [18]. The administration of therapeutically active compounds via nanoparticles is an increasing share of the therapeutics market. The localization of nanoparticles to specific tissues/sites in the body can be modulated by changing particle size and surface chemistry, including the attachment of antibodies or other targeting molecules [19].

Beyond targeting specific tissues and organs, nanoparticles can target specific subcellular locations as well. They typically enter cells via endocytosis and are sequestered in late endosomes/lysosomes. Certain particle physical and chemical properties, such as their size and charge, are often modified to target locations. The ability for the particle to enter the cytosol is often important for many types of cargoes to function. Strategies to achieve this include swelling, fusing or destabilizing the endosomal membrane, increasing the local osmotic pressure to cause endosome rupture [20]. Nuclear targeting of cargoes can also be achieved via conjugation to nuclear localization signal peptides or certain cationic polymers [21]. Similar methods have also achieved mitochondrial localization [22, 23]. Targeting of the endoplasmic reticulum and Golgi apparatus has been reported via certain receptor-mediated retrograde trafficking mechanisms [22, 23].

Nanomaterial size has a demonstrated effect on biodistribution. Certain synthetic polymers, low-molecular weight proteins, and peptides less than 20 kDa in molecular weight exhibit renal tubule biodistribution but are quickly cleared from the body [24, 25]. An overwhelming majority of nanoparticles systemically administered accumulate in the liver and spleen and are cleared via the hepatobiliary system [19, 26]. Nanoparticles smaller than 200 nm in diameter can extravasate through fenestrations in the liver endothelium, often resulting in Kupffer cell uptake. Microparticles, which are above the nanoscale size range (> 1000 nm), can accumulate in the lungs due to physical retention in alveolar capillaries [27]. Many nanomaterials exhibit renal clearance, which is a highly desirable pharmacokinetic property for elimination of potentially toxic metal nanomaterials, such as those used for diagnostic imaging [28]. To be cleared by renal filtration, nanoparticles must be small enough to pass through the glomerular fenestrae (~ 100 nm) and the glomerular basement membrane (GBM) (~ 8 nm pores) [28]. Renal filtration may allow for the imaging of the kidney during the excretion process [29] and to study renal functions [30]. Nanoparticle size can be controlled by several methods, depending on the type of material used [31].

For the treatment of certain conditions such as tumors, nanoparticles are known to accumulate at the disease site passively, by extravasating at sites of dysfunctional vasculature. In tumor-bearing animals, nanoparticles often exhibit tumor localization due to vascular leakiness, also known as the enhanced permeability and retention (EPR) effect [32]. This effect is dependent on particle size [33] and appears to diminish at particles over 200 nm in diameter [34]. There is evidence, however, that this effect may not translate well to human cancers [35].

Nanoparticles can be rapidly removed from the body via hepatobiliary clearance. Kupffer cells are efficient at removing materials that are bound to certain serum proteins—opsins—that mark them for macrophage-mediated phagocytosis as part of the complement activation cascade [36–38]. Nanoparticles can also be taken up by macrophages which migrate to the liver, lymph nodes, and spleen [38], as mediated by the mononuclear phagocyte system (formerly called the reticuloendothelial system). Opsonization can be reduced by altering the surface chemistry of the nanoparticle. The most common method of doing so is by attaching polyethylene glycol (PEG) to the nanoparticle surface, though other methods exist, such as the use of zwitterionic (simultaneously cationic and anionic) polymers [19, 38, 39].

While nanoparticles are mostly designed to exhibit a spherical shape, nonspherical particles can exhibit altered uptake kinetics, subcellular localization, and pharmacokinetic profiles [40]. Shapes such as fibrils, discs, and rods have been synthesized to modulate these biological parameters with respect to drug delivery applications. Methods to control nanoparticle shape include template-based molding (Particle Replication In Non-wetting Templates: PRINT) [41].

A more specific strategy to target diseased tissues, often called "active" targeting, uses molecular recognition elements to coat the nanoparticle, to cause it to bind to cells or tissues of interest. These targeting moieties can include antibodies, peptides, aptamers, and small molecules that can be conjugated to the nanoparticle surface to enhance uptake in target tissues [42]. The targeted delivery of cargoes to particular cell subsets within a target organ could enhance drug therapeutic index [43]. Active targeting by nanomaterials may take advantage of multivalent interactions due to the large number of recognition elements that can be introduced to the surface of a nanoparticle [44]. However, the biodistribution of particles cannot always be shifted by a large degree via the incorporation of targeting moieties. Nanoparticle size, surface charge, and route of administration may play larger roles [4].

Methods for targeting nanoparticles to tissues and disease sites in the body other than the kidneys have been reviewed extensively elsewhere [4, 45]. Further, nanoparticle toxicity/safety can be modified by modulating particle size, chemistry, and payload, to mediate side effects, including immunologic responses [38, 46].

Kidney-targeted nanoparticles

The kidney is the major organ for the maintenance of electrolyte and water balance and to remove waste products from the body. Injurious events including ischemia, toxins, and inflammation can lead to acute kidney injury (AKI), while chronic diseases such as diabetes and hypertension among other can lead to chronic kidney disease (CKD). Few drugs have been approved to treat these kidney diseases, due at least in part to poor pharmacokinetics of drugs with respect to the kidneys [47–50]. To develop new therapeutics for both acute and chronic renal diseases, we believe it is necessary to develop new strategies to increase drug concentrations in renal tissues while at the same time reducing systemic exposure.

To facilitate successful delivery of therapeutics to renal tissue compartments, nanoparticles must overcome various biological barriers before they reach their target location in the kidneys (Fig. 31.1). Systemically administered materials enter the kidney through the glomerular circulation and encounter the glomerular capillaries, which exhibit 70–100 nm wide fenestrations at the endothelium. The GBM is covered by podocytes exhibiting 4–10 nm gaps. For renal filtration, nanoparticles must be small enough to pass through the glomerular endothelial fenestrae (~ 100 nm) and the podocyte slit diaphragm (~ 8 nm pores) [28]. Besides the size barriers, the glomerular endothelium and podocytes are covered by polyanionic surface glycoproteins, while the mesangial matrix and GBM have abundant anionic heparan sulfate. Nanocarriers smaller than 5 nm in size can penetrate the GBM and are filtered into the lumen of the kidney tubules. The apical membrane of the proximal tubules contains protein transporters such as megalin, cubilin, and folate receptor which facilitate the active transport of various endogenous and exogenous molecules into the tubular epithelial cells (TECs) via receptor-mediated endocytosis [51, 52]. Drugs or nanocarriers which are not reabsorbed into the tubules get excreted in the urine. Due to an improved understanding of the interactions of biological systems and the physicochemical properties of nanocarriers, recent progress has been made in delivering therapeutics to various parts of the nephron (Table 31.1).

Glomerular targeting strategies

Many recent studies have suggested potential strategies to localize therapeutics to the nephron (Fig. 31.2) [24, 69–71]. The transport and clearance of nanoparticles and macromolecules across the GBM is governed by their size and charge. In that regard, various strategies have been explored to deliver targeted therapeutics to the glomerulus/GBM of the kidneys. The glomerulus represents a potential target as the mesangial cells of the GBM are directly accessible via the glomerular capillary, which has 70–100 nm [72] wide fenestrations (Fig. 31.2, Route 1). Therefore, nanoparticles between 70 and 100 nm can be designed to extravasate through the endothelial cell layer. Studies with 70, 90, and 100 nm poly(lactic-*co*-glycolic acid) conjugated to biodegradable polyethylene glycol (PLGA-PEG)-based nanoparticles investigated deposition of NPs into glomerular mesangium [53]. They found that 90 nm size NPs showed much stronger kidney localization as compared to the 70 nm and 100 nm NPs. The same group then found that 90 nm PEG-PLGA nanoparticles could successfully deliver dexamethasone acetate (A-DEX) to the mesangium to treat mesangial proliferative glomerulonephritis (MsPGN) [53]. Other studies have highlighted the accessibility of

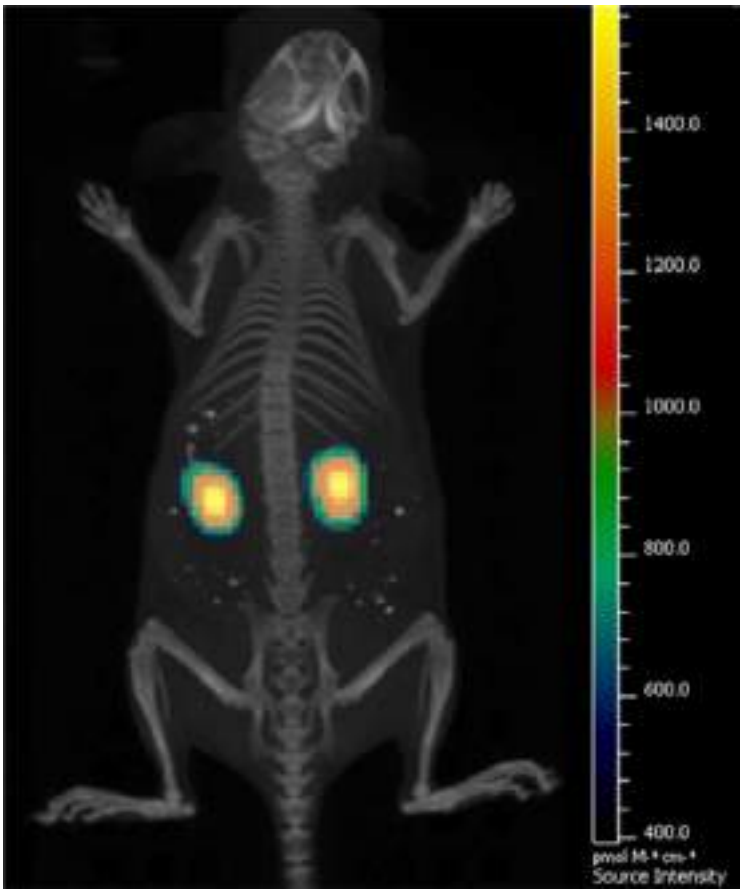

FIG. 31.1 Mesoscale nanoparticles in the kidneys. Fluorescence plus CT overlay focused on the kidneys of a mouse injected intravenously with mesoscale nanoparticles. *Reprinted with permission from Williams RM, Shah J, Ng BD, Minton DR, Gudas LJ, Park CY, Heller DA. Mesoscale nanoparticles selectively target the renal proximal tubule epithelium. Nano Lett 2015;15:2358–64. Copyright 2015 American Chemical Society.*

mesangial cells for the targeted delivery of therapeutics by NPs with hydrodynamic diameter of < 100 nm. One example is the delivery of siRNA via cyclodextrin-based nanoparticles to the GBM [37]. Optical and radiolabeled imaging studies found that these particles localized to and disassembled at the GBM. The same group used a similar system to induce knockdown of green fluorescent protein expression in a reporter mouse strain [73]. Further, they determined that PEGylated gold nanoparticles of ~ 75 nm in diameter deposited into the kidney mesangium [36]. Another group found that fluorescein isothiocyanate (FITC)-labeled albumin nanoparticles (FITC-ANs) with a hydrodynamic diameter of ~ 95 nm displayed significantly higher accumulation in the kidneys after intravenous administration as compared to the albumin nanoparticles with the hydrodynamic diameter of ~ 75 nm or ~ 100 nm. To improve efficacy and to minimize off-target toxicities of celastrol (CLT), a potent immunosuppressant and an antiinflammatory agent, this drug was incorporated into albumin nanoparticles to treat mesangioproliferative glomerulonephritis (MsPGN) [54]. In a rat model of anti-Thy1.1 nephritis, these particles accumulated in mesangial cells and demonstrated alleviation of proteinuria, inflammation, glomerular hypercellularity, and excessive extracellular matrix deposition. Celastrol delivered via albumin NPs significantly reduced accumulation in other tissues/organs as compared to free celastrol in off-target organs and tissues, thereby minimizing celastrol-related systemic toxicities [54].

As the GBM is surrounded by anionic proteins, cationic nanoparticles can be trapped, increasing the deposition of therapeutic cargo to GBM [74, 75]. Positively charged liposomes loaded with prednisolone phosphate demonstrated therapeutic efficacy in a glomerular IgA nephropathy mouse model, significantly reducing antibody and complement deposition in the glomerular mesangium [55]. Further studies demonstrated the successful delivery of different therapeutic cargo, prednisolone and triptolide via cationic liposomes to the mesangial space of the kidneys in mouse and rat models of glomerulonephritis [56, 76].

An alternative approach to target the GBM is via active targeting moieties. One such study engineered perfluorocarbon nanoparticles with collagen IV-targeting ligands on their surface [57]. These studies demonstrated that the intravenously administered particles exhibited significant glomerular deposition as compared to nontargeted

TABLE 31.1 Kidney-targeting nanoparticles.

Type of nanocarrier	Cargo	Physicochemical properties	Target location	Indication	References
Poly(lactic-*co*-glycolic acid) (PLGA-PEG)	DiD, dexamethasone acetate	Size: 90 nm in Shape: spherical	Glomerular mesangium	Mesangial proliferative glomerulonephritis (MsPGN)	[53]
Cyclodextrin	siRNA	Size: 60–100 nm Surface charge: + 10 mV	Glomerular basement membrane (GBM)	NA (Healthy mice)	[37]
PEGylated gold	NA	Size: 75 ± 25 nm Surface charge: − 10 mV	Glomerular mesangium	NA (Healthy mice)	[36]
Albumin	Celastrol	Size: 90 nm Surface charge: − 23 mV	Mesangial cells	mesangial proliferative glomerulonephritis	[54]
PEGylated liposomes	Prednisolone phosphate	Size: 100 nm Surface charge: Cationic	Glomerulus mesangium	IgA nephropathy	[55]
PEGylated liposomes	Triptolide	Size: ~ 100 nm Surface charge: ~−15 mV	Mesangial cells	Membranous nephropathy	[56]
Col4-targeting ligand coupled Perfluorocarbon (PFC) nanoparticle	Prednisone	Size: ~ 200 nm	GBM (collagen 4 receptor)	Lupus nephritis	[57]
Octa-arginine (R8) coated liposomes	p38α MAPK siRNA (p38i) and p65 siRNA (p65i)	Size: ~ 110 nm Surface charge: ~+6.4 mV	Glomerulus	Immunoglobulin A glomerulus nephritis (IgAN)	[58]
Protein-based nanocages	DNA binding protein from starved cells	Size: ~ 12 nm	Proximal tubules	Endotoxin-induced acute kidney injury	[59]
Micelles coated with kidney targeting peptide (lysine-lysine-glutamic acid-glutamic acid)3-lysine ((KKEEE)3K)	NA	Size: ~ 15 nm Surface charge: ~−7.8 mV	Proximal tubules	NA	[60]
PLGA	Oltipraz	Size: ~ 100 nm Surface charge: ~−20 mV	Proximal tubules	Acute kidney injury	[61]
Chitosan	AQP1 siRNA	Size: ~ 200 nm	Proximal tubules	NA	[62]
Carbon nanotubes	Trp53 and Mep1b siRNA	Size: one-dimensional (~ 1 nm diameter, > 300 nm length)	Proximal tubules	Acute kidney injury	[63]
DNA origami nanoparticles (DON)	NA (DNA functions as free radical scavenger)	Size: ~ 90 × 60 nm rectangular DON	Proximal tubules	Acute kidney injury	[64]
PLGA-PEG (mesoscale nanoparticles)	TLR9 antagonist ODN	Size: 311.7 nm Surface charge: ~−25 mV	Proximal tubules	Ischemia reperfusion-induced acute kidney injury	[65]
PLGA-PEG (mesoscale nanoparticles)	Edaravone	Size: 374 nm Surface charge: ~ 23 mV	Proximal tubules	Cisplatin-induced acute kidney injury	[66]
PLGA-PEG (mesoscale nanoparticles)	NF-κB essential modulator (NEMO) peptide	Size: 315.4 nm; Surface charge: ~−25 mV	Proximal tubules	Ischemia reperfusion-induced acute kidney injury	[67]
PLGA-PEG (mesoscale nanoparticles)	Triptolide	Size: 416.9 nm; Surface charge: − 11 mV	Proximal tubules	Ischemia reperfusion-induced acute kidney injury	[68]

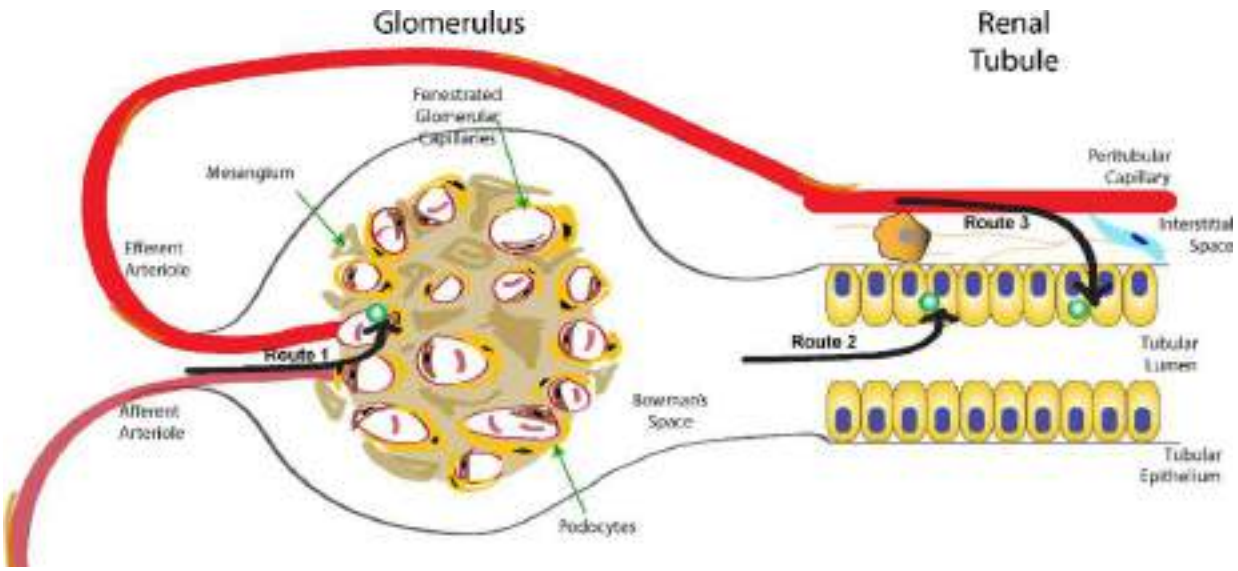

FIG. 31.2 Renal tissue targeting pathways. Depiction of the renal nephron, with the glomerulus on the left and proximal tubule on the right. Three primary routes of renal tissue targeting are highlighted. *Route 1* highlights the primary targeting method for the glomerulus, wherein nanoparticles are filtered out of fenestrated glomerular capillaries and lodge within the foot processes of the podocyte—typically based on size and/or charge of the particles. *Route 2* highlights the classical pathway of tubular targeting, wherein nanoparticles are small enough to be filtered into Bowman's space and are reabsorbed at the tubular lumen of tubular epithelial cells. *Route 3* highlights a strategy for tubular targeting wherein nanoparticles pass from the peritubular capillaries across the tubular interstitium and are taken up via the basolateral membrane of tubular epithelial cells.

controls, as well as therapeutic efficacy following prednisolone encapsulation [57]. Another study found that cationic nanoparticles, functionalized with cell-penetrating peptides, and in the size range between podocyte foot processes and the GBM pores, increased the entrapment efficiency and accumulation of an siRNA cargo in glomerulus [58]. These particles encapsulated siRNA that targets p38α, MAPK, and p65 to treat mice with IgA nephritis. These particles exhibited successful accumulation in mesangial and endothelial cells, efficiently silenced p38α, MAPK, and p65 expression, and alleviated proteinuria, inflammation, and excessive extracellular matrix deposition [58]. These studies suggest that multiple routes are possible to target the glomeruli.

Tubule targeting strategies

TECs reabsorb solutes, electrolytes, proteins, and other compounds into the circulation via an active transport system. TECs are susceptible to various nephrotoxic insults due to drugs, injury, or immune-mediated reactions which can lead to AKI and CKD. Genetic and epigenetic mutation in TECs is also responsible for diseases such as dominant and recessive polycystic kidney disease (PKD) and tubulointerstitial kidney diseases. Therefore, delivering therapeutics to TECs could be of significant clinical value. This has been accomplished via nanoparticle approaches and other protein and/or polymeric approaches, extensively reviewed elsewhere (Fig. 31.2) [24, 70].

One strategy to reach TECs includes glomerular filtration and reabsorption via luminal transporters such as megalin, cubilin, and folate receptors (Fig. 31.2, Route 2). One such study synthesized ~ 10 nm protein nanocages from DNA-binding proteins [59]. Via using intravital microscopy, this work showed that these nanoparticles were filtered through the glomeruli and endocytosed by the proximal tubules, with significantly greater renal deposition than other organs. Moreover, manganese-bound Dps nanocage reduced endotoxin-induced AKI in mice and was well tolerated [59]. In another example, DSPE-PEG (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino-(polyethylene glycol)-based kidney-targeting micellar nanoparticles, ~ 15 nm in diameter size and covalently conjugated to a megalin-binding peptide, exhibited significant renal and hepatic accumulation, safety, and therapeutic efficacy [60].

Tubular targeting may also be possible in diseases which exhibit disrupted glomerular filtration barriers, such as minimal change disease and membranous nephropathy, among others. In these, podocytes undergo flattening and effacement of their foot processes in addition to disruption of the glomerular glycocalyx network [77, 78]. One study explored this accumulation route via PLGA-PEG based nanoparticles [61]. This study investigated the biodistribution of various nanoparticle diameters, finding 100 nm particles accumulated within TECs

significantly more than 200 nm and 300 nm particles in mice with ischemia-reperfusion injured mice. Moreover, these particles exhibited significant therapeutic benefit when loaded with oltipraz compared to soluble oltipraz [61]. In another study, chitosan nanoparticles exhibited delivery of siRNA against cyclooxygenase type 2 (COX-2) to treat unilateral ureteral obstruction (UUO) models of CKD. COX-2 siRNA loaded chitosan nanoparticles were targeted against tubular macrophages. Intraperitoneal injection of COX-2 chitosan/siRNA nanoparticles resulted in therapeutic benefit and reduced COX-2 expression and attenuated oxidative stress/apoptosis [79]. In other work, chitosan nanoparticle-encapsulated siRNA exhibited significant megalin-mediated TEC accumulation compared to lipid-complexed siRNA following intravenous injection [80]. This construct demonstrated up to 50% knockdown of aquaporin 1 in mice [62].

In addition to size and surface charge, particle shape also plays an important role in renal localization. Carbon nanotubes (CNTs) 100–500 nm in length, but only 0.5–2 nm in diameter, rapidly exited circulation via glomerular filtration, but a fraction were reabsorbed by TECs [81, 82]. Follow-up studies demonstrated that these nanoparticles can deliver siRNA, in this case targeting meprin-1β (Mep1β) and p53 to treat AKI [63]. CNT-mediated delivery of siRNA resulted in a 10-fold increase in kidney accumulation within 1 h after injection as compared to control siRNA and significantly improved progression-free survival in cisplatin-induced AKI. Moreover, this platform was found to be safe and well tolerated in mice and demonstrated a comparable biodistribution profile in nonhuman primates [63].

Other studies investigating nanoparticle shape evaluated the renal biodistribution of rectangular, triangular, and tubular DNA origami nanostructures [64]. Radioimaging revealed that all three shaped DONs preferentially accumulated in the kidney tubules, but rectangular nanostructures exhibited minimal liver uptake at 12 h postinjection. Further study found that these particles initially accumulated in the glomerulus and were later found primarily in the renal tubules and they were therapeutically effective in a mouse model of rhabdomyolysis-induced AKI [64].

Nanoparticles can also enter the proximal tubules from the basolateral side of the TECs via transcytosis through peritubular capillaries (Fig. 31.2, Route 3). Selective targeting of the proximal tubules was found to occur with nanoparticles 400 nm in diameter, called mesoscale nanoparticles (MNPs) [19, 71]. These PLGA-PEG spherical nanoparticles localized to the kidneys 26-fold more than any other organ, and they persist in the TECs for several days (Fig. 31.1) [19], although they are much larger than the GBM fenestration. Mesoscale nanoparticles were found to be biocompatible [71] and exhibited therapeutic efficacy against cisplatin-induced AKI when loaded with the free radical scavenger edaravone [66, 83, 84] Further, these particles were therapeutically effective in a mouse model of ischemia reperfusion-induced AKI by delivering an oligonucleotide antagonist of Toll-like receptor 9 (TLR9) to the tubules [65], a peptide that binds to NEMO (NF-κB essential modulator) [67], or a small-molecule natural product triptolide [68].

Nanoparticles in the clinic

The use of nanomedicine approaches in the clinic is rapidly evolving driven by the development of new technologies, delivery strategies, treatment modalities, new drug approvals, and clinical failures of current drugs. In the past few years, several nanoparticles have been approved for use in humans and close to 100 nanoparticle technologies are undergoing clinical trials [85].

Nanotechnologies for cancer

Several clinically approved nanoparticle formulations are currently used for cancer treatment. Among these are included PEG functionalized liposomal doxorubicin [86], liposomal daunorubicin, liposomal vincristine [87], and liposomal irinotecan [88] that were approved by the FDA, whereas non-PEGylated liposomal doxorubicin [89] and liposomal mifamurtide [90] were approved by the EMA. Examples of recently approved nanoparticles include VYXEOS, 100 nm bilamellar liposomes that encapsulate a synergistic molar ratio of cytarabine to daunorubicin of 5:1 and received FDA approval for the treatment of acute myeloid leukemia [91]. The lone nonliposomal nanoparticle system currently approved for cancer treatment is Abraxane, which is an albumin-bound paclitaxel nanoparticle [92].

Another recently approved compound is NBTXR3, a 50 nm crystalline hafnium oxide nanoparticle with negatively charged phosphate coating [15]. NBTXR3 enhances external radiotherapy via a physical mode of action that relies on hafnium's natural radioenhancing properties [15, 93]. NBTXR3 is currently approved for the treatment of locally advanced soft tissue sarcoma and ongoing clinical trials are studying its use in prostate cancer and lung cancer combined with immunotherapy.

Iron-replacement nanoparticle therapies

A clinical area where nanoparticles have made a significant impact is as iron-replacement therapies for the treatment of anemia [94–96]. In these applications, the nanoparticle (iron-oxide colloids) is the therapeutic agent used to increase systemic deposits of iron [97]. These nanoparticle approaches avoid the toxicity associated with free iron infusions [94, 96] that are prevented by using colloidal iron coated with sugars [94, 96].

Nanoparticle/microparticle imaging agents

Iron-oxide nanoparticles are approved as contrast agents for MRI [98, 99]. For imaging applications, the innate magnetic responsiveness of iron-oxide nanoparticles is used with MRI to generate contrast for imaging [100, 101]. The combination of an iron-oxide nanoparticle's MRI responsiveness and small size provides accurate and precise imaging. Interestingly, particles can also be used as intravenous ultrasound enhancing agents that take the form of micron-sized microbubbles [102] which enhance contrast by stabilizing and encapsulating air bubbles [102, 103].

siRNA delivery

Patisiran is a siRNA-delivering lipid-based nanoparticle developed for the silencing of the gene responsible for the expression of transthyretin, which can cause hereditary transthyretin amyloidosis [10]. Patisiran was the first clinically approved example of an RNAi therapy-delivering nanoparticle administered intravenously. Clinical studies of patisiran showed that the serum transthyretin decreased by over 70% in patients receiving as compared to less than 20% in the placebo group. In addition more patients had significant improvements in modified Neuropathy Impairment Score+7 as compared to placebo [10].

Antifungals

Lipid-based nanoformulations have been clinically approved for fungal infections. The antifungal drug amphotericin B, used for treating systemic fungal infections, has been formulated in liposomes (AmBisome) [104].

Conclusions

The large majority of renal drug delivery studies have investigated the applications of nanosized polymer–drug conjugates, liposomes [62, 105–110], or inorganic nano-diagnostic compounds to reduce renal toxicity [111–118]. Although a growing number of studies have reported the utility of nanotechnology approaches for the treatment of renal diseases in preclinical models, to date, there is no nanotechnology currently approved for the treatment of any specific renal disease in humans. Potential applications of nanotechnologies approaches include AKI, glomerular diseases, and perhaps CKD. Few systematic studies of NP glomerular deposition in kidney diseases have been conducted with nanomedicines; however, recent preclinical studies suggest that NPs may be of use for the treatment of kidney diseases. To investigate the therapeutic potential of these technologies in the clinic, IND-enabling studies must be conducted. Additional investment in experimental nanotherapeutics is required to enable these investigations to proceed to clinical trials.

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Disclosures

D.A.H. is a cofounder and officer with equity interest in Goldilocks Therapeutics, Inc., LipidSense, Inc., and Nirova BioSense, Inc. D.A.H. is a member of the scientific advisory boards of Concarlo Holdings, LLC and Nanorobotics, Inc. R.M.W. is a scientific advisor with equity interest in Goldilocks Therapeutics, Inc. E.A.J is a cofounder and chief medical officer with equity interest in Goldilocks Therapeutics Inc. C.K. is an employee with equity interest in Goldilocks Therapeutics Inc.

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CHAPTER

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Small molecules in regeneration

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Acute kidney injury (AKI) is a significant worldwide problem, accounting for almost 20% of all hospitalized patients in the United States [1]. A variety of insults can lead to AKI, including ischemic injury [2], nephrotoxins [3, 4], and sepsis [5]. Several comorbidities have been shown to increase the prevalence of AKI such as diabetes, heart disease, cancer, and immunosuppression [6]. Recent reports have also shown incidents of AKI in hospitalized patients with COVID-19 [7]. Episodes of AKI can result in long-term reductions in renal function, thereby increasing the risk for patients to develop progressive chronic kidney disease (CKD) [8–11]. Incomplete recovery from AKI has been attributed to one of the primary underlying factors contributing to the transition from AKI to CKD. At present, there are no FDA-approved therapies to reduce postinjury AKI. Given then the diverse etiologies that lead to AKI, there are a number of potential pathways involved in regeneration, proliferation, and fibrosis that could be ideal targets for new therapies. Broadly, there are two major classes of therapeutics that are currently being targeted for drug development, small molecules, and biologically based drugs.

Characteristics of small molecule therapeutics

Small molecule therapeutics (SMT), also referred to as small molecule inhibitors, compounds, and small molecule drugs, have been the basis for pharmacological intervention for thousands of years. The rise of organic chemistry in the mid-1800s propelled by the dye industry, most notably Bayer and Sandoz, led the way for today's modern pharmaceutical industry. The first synthetically developed compound, chloral hydrate, was synthesized by a German chemist Justus von Liebig in 1832, and eventually used in the clinic as a sedative-hypnotic in 1869 [12]. Despite the expanding markets for biological-based pharmaceuticals, which comprises nearly 25% of all newly approved drugs between 2015 and 2019 [13, 14], SMT are still the foundation of the pharmaceutical repertoire for treating the myriad of ailments from minor headaches to cancer.

SMT generally have low molecular weights and principally enter cells without the need for specific transporters. Once inside the cell, these molecules can modulate biological processes by targeting proteins or mimicking biological substrates [15]. Pharmacokinetics (PK), the study of how the organism affects the drug, and pharmacodynamics (PD), the study of how a drug affects an organism, are much more predictable in SMT compared to biologics [16]. These properties allow for easier design of new therapeutics and follows less complicated dosage protocols, increasing patient compliance. Compared to modern biologics, SMT also follow the Lipinski rule-of-five criteria, describing the molecular properties or druggability that suggests a drug may be orally active and are important for the absorption, distribution, metabolism and excretion (ADME) of new candidate drugs [17, 18]. Although SMT offer the ease of design, affordability, and compliance for patients, they do have some challenges. Because these compounds are relatively easy to produce, fierce competition for generics can make it difficult for them to be profitable beyond their patent life span, which for most pharmaceutical companies presents a particular challenge. The properties that make SMT advantageous for drug discovery can also lead to potential off-target effects and for drug–drug interactions. However, because biologicals are more complex, they can be specifically targeted to proteins of interest, leading to

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fewer off-target effects, and have the possibility for a longer patent life. But biologics can also lead to immunogenicity [19], cytokine storms [20], encephalopathy, and human neutralizing antibodies against the biologics making them ineffective [21–24].

Small molecule therapeutics targeting for AKI

There are several therapeutic windows to target during AKI events using SMT. Therapeutics could be employed as a preventative measure prior to an AKI event, such as when the injury occurs during scheduled surgical procedures, imaging, or before the induction of antibiotic therapy. These remain the best-case scenarios for the treatment of AKI because these interventional modalities could be used quickly and incorporated into currently used preventative protocols [25]. However, the current challenge with therapeutic intervention for AKI is the development of effective treatments after a sustained injury has occurred. Determining the correct therapeutic window is important because treating with SMT too early could lead to complications or exacerbate the problem [26], whereas treating too late could also have poor outcomes [27]. The alternative treatment window could focus between the onset of inflammation, which helps to improve the outcome of injury through increased proliferation [28] and the induction of productive repair processes [29], but before pro-fibrotic factors begin to drive increased extracellular matrix (ECM) deposition, leading to maladaptive repair [30]. This is one of the primary differences between treatments focused on AKI versus CKD. The goal of drug development in CKD is to target therapeutics that can attenuate further kidney damage. These therapeutics would require long-term, perhaps even life long, interventions to achieve their goal. Not only knowing when to begin therapeutic intervention is important but also understanding which pathways to target is equally as important. In the following section, we discuss only a few of the pathways, which when targeted with SMT could potentially be used for resolution of AKI.

Regeneration

Regeneration is an ongoing process throughout an organism's lifespan. Lower vertebrates and amphibians have an extraordinary capacity for regeneration, but mammals, however, have lost their ability for extensive regeneration [31]. Damage or injury to tissues can lead to apoptosis or necrosis, resulting in a loss of functional cells. Reparative regeneration responds after these insults to help restore the normal homeostasis and function. Even though the mammalian kidney has a limited capacity for regeneration [32], it has the ability to regenerate single cells or small clusters of cells within the nephron [33]. De-differentiation via epithelial-to-mesenchymal transition (EMT), proliferation, and re-differentiation of neighboring cells are able to replace the damaged areas of the nephron [34]. The mammalian kidney lacks the ability to regenerate whole nephrons in the event of severe or prolonged injury such as during sepsis or chronic kidney disease. To enhance the innate ability of the kidney to regenerate, we need to understand the pathways and the cellular factors that are involved in the process. Given the expansive list of regenerative pathways and the limited space, the focus will be on the following several of the most common discussed in the literature, including TGF-β, BMP-7, WNT/β-catenin, and histone deacetylases (HDACs).

Growth factors as targets of small molecule therapeutics in AKI

Growth factors are important signaling molecules that are responsible for stimulating a variety of cellular processes, including proliferation, cell migration, agonists and antagonist of differentiation, and regulation of both cell death and survival. In the last several decades, growth factors have been identified as important factors in a wide range of AKI models, including fibroblast growth factor (FGF) [35], epidermal growth factor (EGF), hepatic growth factor (HGF), insulin growth factor (IGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), and bone morphogenic protein (BMP) (see Gao et al. [36] for excellent review). There is an exhaustive list of growth factors, ligands, and receptors that when activated or inhibited can lead to downstream fibrotic events. Moreover, the type of AKI can heavily influence the part of the nephron that is injured and the specific pathways that become activated during injury [37].

WNT/β-catenin

One of the main pathways that regulates tissue's ability to regenerate is the Wingless-related integration site (WNT) pathway. The WNT pathway is not only one of the key regulating pathways during embryonic development, but it also plays key roles in regulating tissue differentiation, repair, and regeneration during adulthood [38]. There are two signaling cascades regulating the WNT response, the canonical and the noncanonical. WNT ligands bind to frizzled (FZD) and low-density lipoprotein-related receptors (LRPs) to activate their downstream targets [39]. In the absence of WNT, it's transcriptional coactivator β-catenin, is targeted for proteasomal degradation through sequential phosphorylation from a complex comprised of glycogen synthase kinase 3β (GSK3β), adenomatous polyposis coli (APC), casein kinase 1 (CK1), and axin, triggering the ubiquitination of β-catenin [40]. When WNT binds to one of the receptors such as LRP5 or LRP6, one of the downstream intracellular signaling proteins, Disheveled (DVL), stabilizes and prevents the degradation of β-catenin by sequestering GSK3β and its binding partners, allowing β-catenin to translocate to the nucleus and activate its gene targets [41]. The activation of the noncanonical pathway can either increase intracellular Ca^{2+} release or activate the planar cell polarity (PCP). The PCP pathway activation occurs through FZD receptors signaling through Rho-associated protein kinase (ROCK) and c-Jun N-terminal kinase (JNK) leading to cytoskeletal rearrangement and polarization of the epithelial cells [42]. Intracellular Ca^{2+} release is regulated by DVL and G-coupled receptors [43, 44].

WNT/β-catenin in AKI

The WNT pathway is upregulated in the kidney tubules during AKI [45]. In mouse models of AKI using ischemia–reperfusion (IRI) and nephrotoxicity (folic acid), β-catenin activation is upregulated in the tubule cells of the nephron, and there may be a correlation between the level of β-catenin and the severity of injury [46, 47]. It appears that β-catenin is important in the early stages of injury as shown by conditional knock-out studies. Loss of β-catenin in the tubules expressing a kidney-specific protein (KSP/CDH16) after AKI using folic acid or IRI, exhibited more intense injury with a higher mortality rate (50% vs 23.5%) when compared to the control mice. Severity of the injury was increased with induced expression of pro-apoptotic factors Bax and p53, leading to a loss of the brush border in proximal tubules and overall loss of cells [47]. However, prolonged expression of β-catenin can also be damaging to the tubules. This was shown by overexpressing Wnt1 ligand after 30 min of IRI in mice [46]. The sustained β-catenin expression led to an increase in fibrotic genes and protein expression such as α-smooth muscle actin (Asma/Acta2), fibronectin, and increase in collagen deposition suggesting quicker progression to CKD [46]. To show that β-catenin pathway can be modulated, a β-catenin inhibitor, ICG-001(Table 32.1), was tested 5 days after IRI injury and overexpression of Wnt1. Results revealed that 11 days after IRI, there was a reduction in pro-fibrotic gene expression as well as a reduction in collagen deposition in comparison to the vehicle-treated controls. Downstream targets MMP7 and Snail1, both important for EMT, were also suppressed [46]. Recent studies attempted to combine the modulation of two stages of β-catenin action in the context of kidney injury. The first stage increased β-catenin expression with the use of Wnt1, 30 min prior to IRI, acting in a renal protective capacity, while the second stage decreased the expression of β-catenin with the treatment with ICG-001, to stimulate tubule repair. [48] Surprisingly, they found that pretreatment alone with Wnt1, prior to injury, added to the severity of the injury by stimulating deposition of fibrotic factors. However, the combination treatment with both Wnt and ICG-001 or ICG-001 alone improved the outcome by reducing matrix deposition.

Renal tubules are not the only cell types to sustain injury during renal dysfunction. Glomeruli are filtration units within the nephron primarily consisting of podocytes which sit on a basement membrane and contain interdigitating foot processes surrounding the fenestrated endothelial cells creating a filtration barrier. Podocyte injury leads to a leaky filtration barrier and can progress to proteinuria, glomerular disease, and CKD [49]. Wnt/β-catenin is activated after podocyte injury and prolonged expression or hyperactivation leads to severe injury [50–52]. Studies have focused on inhibiting β-catenin using small molecules to reduce podocyte injury. Salidroside (Table 32.1) is a small molecule that has been found in traditional Chinese medicine. One study examined the mechanism of Salidroside in a doxorubicin-induced podocyte injury mouse model [53]. Salidroside was administered immediately after doxorubicin injury and measured 5 weeks later. Results revealed a marked reduction in the fibrotic response with lower collagen deposition, Asma/Acta2 protein, and mRNA expression and prevented loss of podocyte-specific slit diaphragm proteins such as nephrin and podocin. The analysis showed that Salidroside could reduce β-catenin expression and its downstream effectors Axin2 and CyclinD1 [53].

TABLE 32.1 Small molecule therapeutics tested in acute kidney injury.

SMT	Injury model	Target pathway	Source
ICG-001	IRI	B-Catenin	[46, 48]
Pirfenidone	Gent-AKI	TGF-B	[76]
Telbivudine	UUO	TGF-B	[71]
Asperulosidic acid (ASPA)	UUO	TGF-B	[53, 72, 73]
Salidroside	Podocyte injury	WNT	[53]
18b-Glycyrrhetinic acid	CP-AKI	HDAC	[127]
G200745	UUO	HDAC	[104]
MC1568	UUO	HDAC	[105, 106]
Piceatannol	UUO	HDAC	[104]
MS-275 (Entinostat)	IRI, CP-AKI, S-AKI	HDAC	[110, 112, 128]
TMP195	S-AKI	HDAC	[138]
Trichostatin A (TSA)	UUO, IRI, CP-AKI	HDAC	[102, 110, 112, 126, 128]
Tubastatin A	CP-AKI, S-AKI	HDAC	[128, 129, 142]
4-(Phenylthio)butanoic acid (PTBA and prodrugs UPHD25, UPHD186)	Gent-AKI, AAN, UUO, IRI, S-AKI	HDAC	[26, 27, 144–146]
Romidepsin (FK228)	CP-AKI, S-AKI	HDAC	[128, 140]
Suberoylanilide hydroxamic acid (SAHA, Vorinostat)	IRI	HDAC	[134]
Valproic acid (VPA)	IRI	HDAC	[126]

AAN, aristolochic acid nephropathy; *CP-AKI*, cisplatin AKI; *Gent-AKI*, gentamicin AKI; *IRI*, ischemia reperfusion injury; *S-AKI*, Sepsis AKI; *UUO*, unilateral ureteral obstruction.

Transforming growth factor-β pathway

TGF-β is an important cytokine that helps to maintain normal cellular homeostasis through control of cell growth, proliferation, differentiation, wound healing, and immune functions. TGF-β is part of the superfamily of related growth factors that are secreted in a latent form as a propeptide (LAP). The activation of latent TGF-β is a major regulatory step controlling the effects of TGF-β, as the latent TGF-β is activated through proteolysis from plasmin and matrix metalloproteases (MMP), binding to integrins, mechanical forces due trauma, or binding to thrombospondin-1 (TSP-1). TSP-1 is thought to control the activation of TGF-β during stress and pathological conditions. In diabetic type 1 model of nephropathy in mice, blocking TSP-1 reduced real injury [54–56]. There are three isoforms of TGF-β in mammals, TGF-β 1, 2, and 3, which have all been identified in the kidney [57]. TGF-β-1 is the only of the three that appears to play a role during AKI events [58].

TGF-β ligands normally bind to type I or type II receptors, and trigger either canonical (SMAD-dependent pathway) or noncanonical (SMAD independent) pathways setting off a cascade of reactions leading to the increase of target genes in the nucleus. The noncanonical TGF-β pathway is mediated through Ras–Raf–MEK–ERK pathway, TGF-β-activated kinase 1 (TAK1), and phosphatidylinositol-3 kinase (PI3K)-AKT pathway [59]. Our focus will be on the SMAD-dependent pathway, but Zhang [59] has an excellent review on non-SMAD-dependent pathways.

In the canonical pathway, TGF-β ligands bind to TGF-β type II receptors, including TGFBR2, BMPR2, ACVR2A, ACVR2B, and AMHR2. Activated TGFβ-1 ligand binds to TGFBR2 inducing the heterodimerization with TGF-β type I receptors, initiating the phosphorylation of downstream mothers against decapentaplegic homolog (SMAD), SMAD2 and SMAD3. Bone morphogenetic protein (BMP) ligands, another TGF-β family member, binds to TGFBR2, which initiates the phosphorylation of the SMAD1/5/8 complex. Both SMAD2/3 and SMAD1/5/8 recruitment of SMAD4 aids in the nuclear translocation of the SMAD complex. SMADs are important mediators of the TGF-β signaling pathway and have been implicated for their role in kidney fibrosis [60–64]. Opposing roles of SMAD2 and SMAD3 were identified in renal fibrosis. Mice deficient in SMAD3 were found to be protected against tubulointerstitial fibrosis following unilateral ureteral obstruction (UUO) [65]. Expression of collagen I, collagen III, and tissue inhibitor of metalloproteinases 1 (TIMP-1), which are essential factors during EMT, was increased in SMAD2 knockout

mice after UUO [62]. siRNA of SMAD4 was found to inhibit renal fibrosis in folic acid-induced mouse model of AKI [66], and conditional deletion of SMAD4 inhibits fibrosis by suppressing SMAD3 promoter activity and preventing the binding of SMAD3 to the COL1A2 promoter [67]. While TGF- β activates pro-fibrotic factors, BMP may be renal protective. Kidney injury induced by gentamicin in rats activated the TGF-β-SMAD2/3 pathway but suppressed the SMAD1/5/8 pathway [68]. BMP-7 which is a specific isoform identified in the kidney activates SMAD1/5/8 and promotes the repair of kidney injury in mice following resolution of UUO [69]. Exogenous treatment with BMP-7 in mice following injury with UUO increases GFR and decreases renal atrophy [70].

Elevated levels of circulating TGF-β in mice developed glomerulosclerosis. In models of unilateral ureteral obstruction (UUO), TGF-β signaling factors were downregulated after treatment with Telbivudine (LdT) (Table 32.1), an inhibitor of the TGF-β pathway [71]. Asperulosidic acid (ASPA) (Table 32.1), a herb that is commonly found in regions of Asia, has been used for the treatment of nephritis [72], and has been shown to have antiinflammatory properties [73]. In rats with UUO, ASPA decreased the expression of TGF-β-1, SMAD2, SMAD3, and SMAD4, in addition to the reduction of α-SMA, collagen II, and fibronetin [74]. In tubular cells, TGF-β induces basement membrane thickening via increase of ECM expression, cell hypertrophy, cellular apoptosis, and EMT [75]. Recently, Pirfenidone, which decreases the production of TGF-β and is currently approved by the FDA for the treatment of idiopathic pulmonary fibrosis, was used in the treatment of gentamicin-induced AKI in rats. Pretreatment for 7 days before induction of AKI improved renal function through decreasing oxidative stress and attenuating the activation of the nucleotide-binding oligomerization domain [NOD]-like pyrin domain containing protein 3 (NLRP3) inflammasome [76].

Posttranslational modifications (PTM) influence on factors in fibrosis

SMADs, like many cellular mediators, are subjected to various PTMs that modify their activity. These modifications can include phosphorylation, ubiquitylation, SUMOylating, and acetylation, through the activity of HATs and HDACs [77]. SMAD2/3 require phosphorylation for activation and entry into the nucleus through binding of SMAD4. However, in HaCaT cells, SMAD2/3 cycling between the nucleus and cytoplasm was observed through continuous low-level dephosphorylation in the nucleus, where SMAD2/3 was transported to the cytoplasm and rephosphorylated before complexing with SMAD4 and translocated back to the nucleus [78]. While shuttling of SMAD complexes are achieved through modulation of phosphorylation, degradation of SMAD3/4 is facilitated by ubiquitination and subsequent transport to the proteasome. Rodents with antithymocyte serum nephritis were found to have an increase of both ubiquitination and degradation of SMAD2 [79] indicating the important function of protein turnover in kidney dysfunction. Transcriptional activation of SMAD3 and SMAD4 are regulated through acetyltransferase activity of p300 [80, 81]. SMAD4 acetylation in breast cancer cells enhances formation of the SMAD complex in the cytosol and increases nuclear translocation of the complex [82] whereas SMAD3 acetylation enhances transcriptional activity [81].

Histone deacetylases

Histone deacetylases (HDACs) were first identified by their function in deacetylating the lysine residues on histone tails, resulting in epigenetic modifications to gene expression [83]. They are vital enzymes identified in organisms from bacteria to humans [84]. In humans, there are 18 HDAC enzymes divided into four classes, based on sequence and structural similarities [85]. Class I contains HDACs 1, 2, 3, and 8. Class IIa contains HDACs 4, 5, 7, and 9 and class IIb contains HDACs 6 and 10. Class IV consists of HDAC11, which is structurally unique to all other HDACs. Class I, II, and IV share a highly conserved zinc catalytic domain, while Class III, the sirtuins, are NAD^+ dependent.

There are two distinct but overlapping targets of deacetylation involving HDACs. Traditionally, HDACs were thought to target epigenetic changes along the lysines of histone that tails to modify chromatin, and thereby alter gene transcription. In recent years, it has been shown that PTM of nonhistone proteins modified by acetylation may be as important as phosphorylation PTM events [86]. Acetylated proteins regulate protein stability, enzyme activity, and control protein–protein interactions, ultimately regulating important cellular process such as DNA damage repair, signal transduction, autophagy [87], and cell division [88, 89]. Despite its well-known role in the alteration of chromatin accessibility, HDAC inhibitor (HDI) treatment produces global acetylation alterations to the proteome, while modifying gene expression by only 2%–10% [90, 91].

HDACs have been shown to play roles in both the nucleus and the cytoplasm [89]. Class I HDACs are typically located in the nucleus, but class II HDACs shuttle between the cytoplasm and the nucleus [92]. Several HDACs have

TABLE 32.2 FDA-approved histone deacetylase inhibitors.

Drug	Target	FDA-approved treatment	Date of approval
Belinostat (PDX 101)	Pan	Peripheral T-cell lymphoma	2014
Panobinostat (LBH-589)	Pan	Peripheral T-cell lymphoma	2015
Romidepsin (FK228)	Class I	Cutaneous T-cell lymphoma	2009
Vorinostat (SAHA)	Pan	Cutaneous T-cell lymphoma	2006
Valproic acid	Class I, IIA	Epilepsy, migraines, bipolar disorder	1978

been identified in the kidney, with HDAC1 and HDAC2 identified across all cell types [93]. Five classes of HDIs have been described based on their common pharmacophore models consisting of a zinc binding group (ZBG) also referred to as the warhead, a cap, and a linker that connects the cap to the ZBG. Hydroxamic acids, cyclic peptides, benzamides, short-chained fatty acids, and ketones [94, 95] primarily act through binding to the Zn^{2+} containing catalytic domain of Class I, II, and IV HDACs [96] whereas the Sirtuins are NAD^{+}-dependent HDACs that are inhibited by NAD and its derivatives, most notably Sirtinol, Splitomicin, and dihydrocourmarin [97]. Currently, the FDA has approved only five HDIs for use in humans (Table 32.2). Vorinostat (suberoylanilide hydroxamic acid/SAHA) was the first HDI approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma. [98] Panobiostat (LBH-589) was approved by the FDA in 2015 for multiple myeloma, Belinostat (PDX-101) in 2014 for peripheral T cell lymphoma, and Romidepsin in 2009 for cutaneous T-cell lymphoma and peripheral T-cell lymphoma [99]. Valproic acid was first approved for use in France in 1967, and is the most widely used antiepileptic drug in the world [100]. It has also been approved for treatment of migraines and bipolar disorder.

HDAC inhibitors in various models of AKI

Unilateral ureteral obstruction (UUO) is a common model used to induce renal fibrosis and is triggered through oxidative stress, inflammation, and fibroblast activation through the obstruction of the ureter and reflux of urine into the kidney [101]. UUO upregulates both class I and class IIa HDACs. Trichostatin A (TSA) (Table 32.1), a non-competitive reversible hydroxamic acid that inhibits class I and class II HDACs, was shown to inhibit renal fibroblast activation by attenuating STAT3 in UUO [102]. Recently, structural studies of the zebrafish HDAC6 catalytic domains in complex with either the (S) or (R) enantiomers of TSA was shown to either have specificity for HDAC6 or was a broad range inhibitor for Class I or class II HDACs, respectively [103]. G200745 (Table 32.1), which is a pan hydroxamic acid HDI, has also shown efficacy in decreasing NGAL, a known biomarker of kidney injury, after UUO when given immediately following injury. HK-2 cells that were exposed to TGF-β to induce fibrosis, also had lower levels of P-SMAD2/3 and SMAD4 compared to untreated cells disrupting the normal path of pro-fibrotic factors [104]. The hydroxamic acid MC1568 (Table 32.1) is one of the few identified selective class II HDACi [105]. In a delayed treatment model of UUO, where MC1568 was administered 3 days after the initial insult, expression of fibrotic markers fibronectin, collagen-1, and α-SMA were reversed compared to untreated animals. Analysis of renal tissue also showed increased expression of BMP-7, SMAD7, and Klotho [106], suggesting that HDI may work along multiple pathways as a reno-protective mechanism. In another mouse model of UUO HDAC1, 4, 5, 6, and 10 were upregulated in the kidney. Treatment with twice daily injections of piceatannol for 2 weeks was only effective at decreasing HDAC4 and HDAC5. Phosphorylation of p38-MAPK was markedly decreased in response to piceatannol, as was collagen type I, fibronectin, and a SMA. Interestingly, the pro-fibrotic pathway of TGF-β with Smad2/3 was unaffected by piceatannol [107].

Ischemia reperfusion injury

Ischemia reperfusion injury (IRI) is a significant contributor to AKI. Insults from trauma, sepsis, circulatory arrest with resuscitation, vascular and cardiac surgery, and kidney transplantation can lead to IRI [108]. During the initial restriction of blood flow, organs experience severe hypoxia, followed by restoration and perfusion leading to reoxygenation. Tubules undergo apoptosis, necrosis, hypoxia, inflammatory cascades, and activate innate and adaptive immune responses [109]. Class specific and pan HDI were found to increase survival and decrease fibrosis in both

warm and cold ischemia models in mice [110]. Animals were pretreated with TSA (Pan HDI) or MS-275 (Class I HDI) (Table 32.1) for 16 h prior 28 min of ischemia followed by contralateral nephrectomy. TSA or MS-275 treated mice had lower BUN, inflammatory markers, fibrosis, and increased survival after 30 days. The same group used the cold ischemia IRI transplant model to recapitulate the cold ischemia that organs experience before transplantation. Donor and recipient mice were pretreated with TSA prior to transplant. Kidneys were removed from the donor mice and subjected to cold ischemia for 18 h before transplant into the donor mouse. Native nephrectomy was performed 7 days after transplant to overcome the severe graft dysfunction after the prolonged IRI. HDI-treated mice showed less fibrosis and improved renal function. However, the authors note the cold ischemia-treated mice did not have a decrease in inflammatory and heat shock proteins that was noted in the warm ischemia model, indicating the differences in pathways effected by different injury settings. One of the ongoing challenges of therapeutic drug testing in mammals is the known differences in drug metabolism in rodents verses humans. Rodents have a much faster metabolism and require higher doses for longer durations to achieve the same or similar effect to humans [111]. To mimic the pharmacokinetics of humans, Hyndman et al. used osmotic mini-pumps for continuous delivery of either vehicle, MS-275, or TSA for 3 days prior to induction of IRI in mice. Proximal tubule cells had a fourfold increase in HDAC4 expression after IRI, and a two-fold increase in HDAC1. Treatment with either TSA or MS-275 did not result in a significant change to the expression of either HDAC1 or HDAC4. They did identify a reduction of in vivo proliferation in response to TSA, but not MS-275. Additionally, their findings showed an increase in creatinine along with an increase in kidney damage with HDACi treatment. This supports ongoing conflicting data regarding HDACi, where the benefits of HDACi may be effective in only certain types of kidney injury. The authors suggest these data, point to a class II isoform specific mechanism of proliferation and repair mechanisms through HDAC inhibition [112].

Cisplatin

Cisplatin (CP) is a commonly used platinum-based chemotherapy drug used to treat head and neck, lung, testis, ovary, and breast tumors. One of the major side effects is the serious induction of nephrotoxicity leading to AKI. Between 20% and 30% of patients will develop AKI after administration of CP [113–115]. In rodent models, active transport of CP is mediated by copper transporter-1 (Ctr1) [116], organic transporter2 (OCT2) [117, 118], and multidrug toxin extrusion 1(MATE1) [119]. Cisplatin is activated intracellularly through aquination, with the displacement of two chloride ions with two water molecules. This highly active platinum compound interacts with DNA, forming inter and intra-strand crosslinking that inhibits transcription and DNA repair [120, 121]. In the kidney, CP leads to necrosis, apoptosis, and necroptosis [122–124]. These mechanisms are triggered through DNA damage, damage to ER and mitochondria, oxidative stress, and inflammatory cytokine upregulation. HDAC2 was identified as an epigenetic modifier of BMP-7 [125] in IRI. CP-AKI increases expression of HDAC2 and chromatin immunoprecipitation (ChIP) confirmed the upregulation of BMP-7 expression through epigenetic regulation by HDAC2. Pretreatment with either TSA or valproic acid (VPA) (Table 32.1) decreased apoptosis and increased BMP-7 [126]. The medicinal HDI, 18β-glycyrrhetinic acid (Table 32.1), the active component in licorice and known to inhibit HDAC2, was also found to increase BMP-7 and decrease apoptosis [127]. CP-AKI has also shown to increase mRNA expression of HDAC1, HDAC2, and HDAC6. ChIP analysis showed increased acetylation of histones at the promoter region of the microglia/macrophage WAP domain protein (AMWAP), an antiinflammatory and antiapoptotic protein. Pretreatment with TSA prior to CP-AKI was found to suppress inflammation and decrease apoptosis in increased expression of AMWAP in renal tubule epithelial cells. Further analysis of AMWAP in TKPTS, a murine proximal tubule cell line, treated with either MS-275 (HDAC1&3), Romidepsin (HDAC1&2), or Tubastatin A (HDAC6) (Table 32.1) showed increased expression of AMWAP, with Tubastatin A showing the least increase overall. These data suggest that both class I and class II HDACs can regulate inflammation and apoptosis through AMWAP [128]. Increased activation of HDAC6 has been shown in CP-AKI, but inhibition with Tubastatin A has also been shown to improve kidney function, as noted by decrease in NGAL, and decrease apoptosis [129]. One of the major drivers of apoptosis in CP-AKI is the activation of p53 [130–133]. Pretreatment with SAHA (Table 32.1) or TSA (class I and II pan-HDIs) has been shown to suppress CP-AKI-induced apoptosis by attenuating the DNA damage response and p53 activation [134].

Sepsis

Sepsis is the leading cause of death in hospitalized patients in intensive care units. Sepsis-induced AKI (S-AKI) accounts for 50% of the total number of AKI cases [135]. Sepsis begins through an infection, which progresses as the pathogens or toxins enter the bloodstream leading to a systemic inflammatory response. There are two commonly

used methods for testing S-AKI in animal models. The first is the use of lipopolysaccharide (LPS) which binds directly to toll-like receptors (TLRs) beginning the cascade which induces S-AKI. The second is the cecal ligation and puncture (CLP) is the gold standard in sepsis research [136]. Sepsis develops in the peritoneal cavity because the compromised cecum allows gram-positive and gram-negative organisms entry into the normally sterile abdominal cavity. Sepsis is driven by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPS) which interacts with TLRs releasing metalloproteinases 2 (TIMP2) and activating NK-kB, releasing cytokines such as IL-1B, IL-6, and TNF-A [137]. The induction of cytokines and subsequent binding to receptors triggers downstream pathways that lead to apoptosis. Several HDIs have demonstrated reno-protective properties in the LPS S-AKI model. Pretreatment for 5 days with MS-275 was shown to inhibit ROS, decreased ER stress, and increased survival in mice challenged with LPS to induce S-AKI [138]. LPS exposure was shown to upregulate proinflammatory cytokines TNF-A and IL-1B, increase pro-apoptotic factors, while decreasing expression of BCL2 and BMP-7. Mice treated with a selective class IIa HDAC inhibitor (TMP195) (Table 32.1) had lower HDAC4 activity as noted by a decrease in acetylated histone H3K14, lower markers of apoptosis, an increase in BCL2 and BMP-7, and a decrease in proinflammatory cytokines. Overall, the protective effects of TMP195 were demonstrated with an increase in renal function as shown through reduction of NGAL, Kim1, serum creatine, and BUN [139]. The cyclic tetrapeptide Romidepsin, which is a selective HDAC1 and HDAC2 inhibitor decreases the expression of Cytochrome P450 2E1 (CYP2E1) [140]. Reactive oxygen metabolites are generated by CYP2E1, and CYP2E1 −/− null mice were found to be protected against nephrotoxicity and apoptosis [141].

In a study using CLP in rats, HDAC6 inhibition with Tubastatin A attenuated renal injury by decreasing PHB1, an important protein involved in the maintenance of the respiratory chain of the mitochondria [142]. They also identified an increase in peripheral blood mononuclear cell (PBMCs) levels of HDAC6 in patients with sepsis. These studies also suggest that targeting a specific isoform of HDAC could serve as a therapeutic target in S-AKI.

Most AKI treatments are preventative, treating just before or at the same time injury is induced. However, a recent study focused on interventional treatment after S-AKI, which would be more clinically relevant. Mice were either treated 48 h post-CLP or 96 h post-CLP. When mice were treated 48 h after CLP with an HDI, there was an increase in pro-fibrotic gene expression, accompanied by increasing pro-inflammatory cell phenotypes and decreased mononuclear cell infiltration [26]. However, when mice were treated 96 h post-CLP, the HDI prevented fibrosis and improved survival. The authors concluded HDI intervention is best when delayed beyond the initial damage window (48 h post-CLP treatment), and treated during the repair phase (96 h post-CLP treatment).

Interventional models

4-(Phenylthio)butanoic acid (PTBA) (Table 32.1) treatment as either it's methyl ester (m4PTB/UPHD25) or benzamide (UPHD186) prodrug has been shown to be effective in multiple models of AKI. PTBA was originally determined to have HDI activity and to expand the renal progenitor population in zebrafish through a phenotypic screen [143]. Hypothesizing that PTBA could be a therapeutic candidate for kidney injury, the same group tested its efficacy on kidney regeneration in a gentamicin-induced AKI (Gent-AKI) model in larval zebrafish where treatment was found to increase survival and proliferation of renal tubular epithelial cells [144]. Administration of m4PTB, a methyl ester analog of PTBA, 24 h after a uninephrectomy followed by contralateral IRI in mice improved creatinine at 3 and 5 days after injury, and had a reduction in mRNA levels of *Collagen 1* and *LoxL2*, both markers of fibrosis [144]. Next, they examined the effects of m4PTB in a severe model of IRI using unilateral IRI with contralateral nephrectomy 8 days after injury, with m4PTB treatment 24 h after injury. Similarly, mice treated with m4PTB had reduced levels of creatinine and BUN, 9 days after injury. mRNA of *Kim1*, *Collagen 1 Lox*, *LoxL2*, and *a-SMA* were markedly decreased in m4PTB-treated mice. Similar results were observed in treatments with m4PTB after injury with aristolochic acid, which was shown to decrease tubule injury and reduce fibrosis [145]. Using their established phenotypic screen in zebrafish, Skrypnyk et al. [146] identified a prodrug of PTBA, UPHD186, that when metabolized produces the active analog PTBA. They were able to confirm the postinjury reduction in fibrosis after treatment following injury with either UUO or IRI. They also induced IRI with 32 min of clamp time and after 30 days obtained a biopsy to determine the extent of injury. Mice were treated with UPHD186 for 10 days following biopsy confirmation and were harvested 50 days after onset of injury. Despite treatment with UPHD186, there was no change in fibrosis markers compared to vehicle-treated mice. The authors suggest that the window of treatment is only effective when UPHD186 is administered after the onset of regeneration but before fibrosis has been established [146].

Future of small molecule inhibitors

In AKI, use of SMT are still in the early stage of preclinical research (Table 32.1). The field of oncology has developed SMT for decades that target a large array of molecular pathways that have been successfully used in the treatment of a wide range of cancers [147]. Understanding the complex molecular mechanisms of specific types of injury in addition to understanding the implications of comorbidities may be an important component to the development of targeted SMT for treating specific types of AKI. Indeed, a recent study incorporated artificial intelligence with available laboratory measurements, vital signs, and comorbidities to identify specific subphenotypes of septic AKI [148]. Others have also used retrospective studies for determining the subphenotypes in response to vasopressin therapy [149]. Incorporating these types of data with ongoing investigations into the molecular mechanism of AKI from specific types of injury will improve targeting productive, rather than nonproductive repair through regenerative pathways. By far, HDIs have been at the forefront of current research for the treatment of AKI. They have been used successfully for the treatment of epilepsy, migraines, manic-depressive disorder (Depakote), and varying forms of malignancies (Vorinostat, Belinostat, Panobinostat, and Romidepsin). In part because of their current FDA approval (Table 32.2) and previous studies in humans, HDIs may be a good treatment option to investigate in AKI because their known side effects and toxicities have already been established. Despite these favorable attributes, there have been no FDA-approved HDIs for use in AKI, which may be in part, due to several HDIs displaying significant side effects [150]. During phase 1 clinical trials for treatment of advanced acute leukemias with Belinostat (PXD101), dose-limiting toxicities included hypoalbuminemia, hypocalcemia, and elevated creatinine and AKI [151]. Other early clinical trials with Vorinostat (SAHA) [152] and Romidepsin [153] were shown to increase creatinine and cause anemia and thrombocytopenia. Romidepsin in particular was found to be contraindicated for patients with pre-existing cardiac comorbidities, as it was found to increase the QT interval in several patients, leading to on-study deaths [154]. However, the driving factors between a positive outcome in AKI compared to treatment for cancers, are the PK/PD and specificity of the HDIs. Treatment for AKI will likely require lower doses, and shorter duration of treatment compared to the aggressive anticancer treatments currently used. Understanding how HDACs function in specific injury settings of AKI will also be important for the proper application of isoform-specific inhibitors [155].

Among other SMT is Pirfenidone (Table 32.1), a TGF-β inhibitor that has been used for the treatment of idiopathic pulmonary fibrosis. Current studies examining its effectiveness in AKI have not been very successful. Phase 2 clinical trials for Pirfenidone are ongoing for the prevention of CKD in patients who are at risk of progression to end stage renal disease (ESRD) and in FSGS, where early results have been promising, showing a decrease in creatinine over a 12-month period.

Small molecule inhibitors, particularly those that target TGF-β, WNT, and HDAC pathways are promising avenues for treatment of acute kidney injury. Further mechanistic studies are needed to evaluate potential targets in regenerative pathways and understanding the variation of injury will be important incite for the future design and implementation of SMT for the arsenal in combating AKI in the clinic.

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CHAPTER

33

Systems biology in diagnosis and treatment of kidney disease

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Introduction

The kidney is a complex organ responsible for maintaining homeostasis in the human body. In health, the kidney contains over 20 different cell types, which are precisely arranged in a complex spatial configuration. As regenerative medicine is the study of repair, replacement, or regeneration of cells, it quickly becomes apparent that the kidney is an impressively complex and dynamic system to study. For this reason, applying a more holistic approach like systems biology to answer questions in regenerative nephrology is inherently appealing.

What is systems biology?

The term "systems biology" has evolved over time and has been defined in numerous different ways [1]. We define systems biology as the use of computational modeling and mathematical techniques to develop an integrative picture of a biological system built from multiple types of data [2]. In its infancy, systems biology could involve examining the association between one gene and one phenotype. With the maturation of computational approaches and an explosion of high-throughput molecular interrogation technologies, systems biology approaches in nephrology now integrate high-dimensional molecular data from multiple technologies with clinical and histologic data. These data types are integrated using a variety of computational techniques, including data reduction methods, clustering, and machine learning algorithms to extract new biological insights [2].

Several observational studies are now collecting a variety of biospecimens, including kidney tissue, blood, and urine for molecular evaluation [3–6]. With the advent of novel molecular interrogation techniques and systems biology approaches, these various data types from a rich array of biosamples can be integrated to provide insights into the complex, interconnected biological networks involved in kidney health and disease. This will ultimately increase our ability to better differentiate disease pathways and provide tailored prognosis for individual patients (Fig. 33.1). Importantly, this research has also improved our understanding of kidney health, development, and response to injury.

The focus of this chapter is on key computational strategies, salient features, and challenges relevant to dealing with high-dimensional data, i.e., comprising numerous features or variables. We then review the various molecular interrogation technologies and demonstrate how systems biology approaches relevant to kidney function and disease have benefitted the field of nephrology.

Essential concepts in systems biology

Data reduction strategies

Systems biology often involves high-throughput technologies that generate "big data" comprising hundreds or thousands of features, such as genetic variants, transcript expression levels, and protein levels from each sample.

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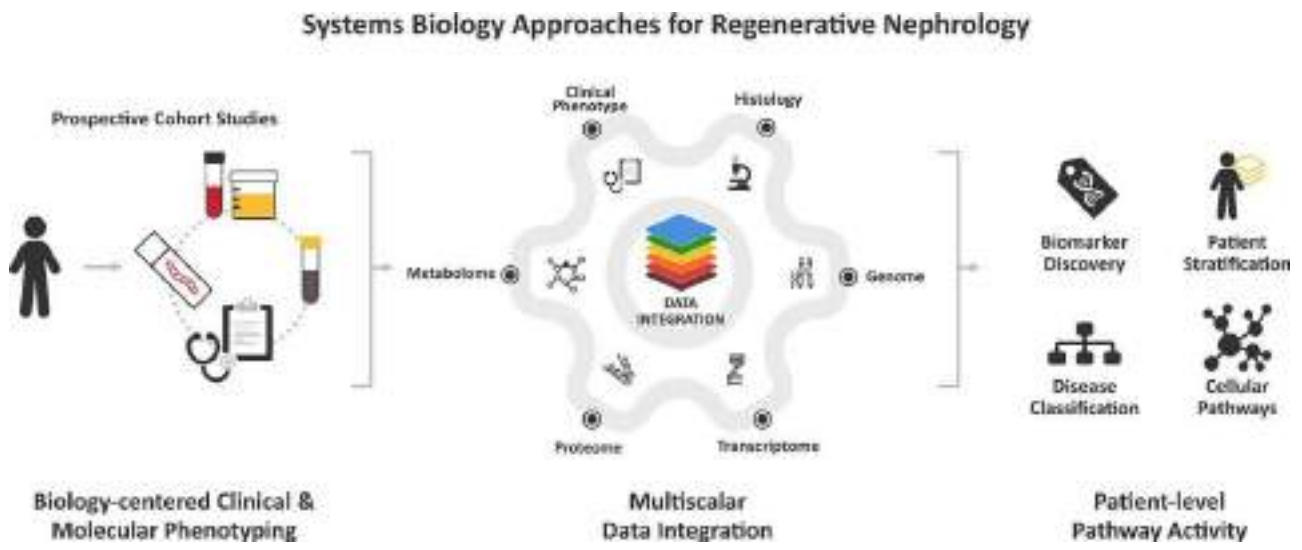

FIG. 33.1 Systems biology integrates multiscalar data from patient cohorts and can be used for biomarker discovery, disease classification, patient stratification or identification of cellular pathways.

As these features are biologically related to one another, data reduction methods attempt to mathematically collapse these data into manageable groups in biologically meaningful ways [7]. One mainstay in the systems biologist's computational toolbox is Principal Components Analysis, which seeks to identify the ideal combination of hundreds or thousands of variables into orthogonal components (i.e., a principal component) [7]. The first principal component will explain the largest amount of variability in the data, the second principal component will explain the second largest amount of variability in the data and will be orthogonal to the first principal component and so on. Principal components analysis is often an essential first step when analyzing "big data" as it elucidates the underlying structure in the data.

Adjustment for multiple corrections

The accuracy of "big data" analyses methods were initially met with some skepticism because thousands of statistical tests were performed on relatively small sample sizes. Standard methods correcting for the error rate, such as the Bonferroni method, were far too conservative and greatly diminished statistical power. Subsequently, novel methods were developed for correction; one of the most commonly used is the Benjamini-Hochberg method to decrease the false discovery rate [8]. This method rank orders results from most to least statistically significant and selects the top ranking *P*-values. This method reduces type I statistical error (i.e., incorrectly rejecting the null hypothesis), while preserving statistical power (i.e., rejecting the null hypothesis if it is false).

Ontologies

To explore relationships within "big data," systems biology borrows from a branch of philosophy studying the nature of entities and their relationships called ontologies. This concept has now morphed into a powerful and indispensable tool whereby entities and their relationships within the data are used to create computer readable ontologies [9]. To make information computer readable, an ontology relies on a controlled vocabulary or standardized definitions, hierarchies, and semantic relationship between the entities. While controlled vocabularies and ontologies are closely linked, ontologies have additional capabilities. For example, while a controlled vocabulary enables retrieval of data within a database, the semantics and relationships of an ontology can query the data to make discoveries. For example, if a patient has glomerular basement membrane thickening on a kidney biopsy, and glomerular basement membrane thickening has been annotated as a feature of diabetic nephropathy in an ontology, the ontology can be used to infer that the patient may have diabetic nephropathy.

Ontologies are rapidly expanding and have now become essential tools used in biomedical research, including the gene ontology, protein ontology, cell ontology, human phenotypes ontology to name just a few [10–13]. These ontologies underlie much of modern biomedical research, but their implementation is often invisible to the user. Many of the computational approaches used in systems biology would not be possible without an ontology to formally

represent preexisting biologic knowledge in a computer readable fashion. Novel applications of ontologies are helpful for diagnosing patients with rare diseases, and there is ongoing effort to improve the utility of ontologies for the nephrology community [14, 15].

Publicly available large-scale datasets and open-source software

To accelerate research and reduce costs, the advent of "big data" in biomedical research has propelled the research community to publically release data. Many investigators now voluntarily post their data on their laboratory's website, and more importantly, in structured data-sharing interfaces. The National Institutes of Health now oversees the access to numerous datasets, including gene expression data in Gene Expression Omnibus, genetic data in the Human Genome Browser, metabolome data in Metabolomics Workbench and many others [16, 17]. While public release of data is a laudable goal for scientific sharing and openness, simply sharing data publically does not ensure that the data will be useful for other investigators. Many publicly available data repositories now require that data meet FAIR principles (Findable, Accessible, Interoperable, and Reusable) (Table 33.1) [18]. These principles ensure that data are shared meaningfully and include necessary detail so the data is usable by other researchers.

Parallel to the push to make large-scale data sets publically available, analytical tools to interrogate these data have been developed as open-source software. Open-source software makes source code publicly available and allows users to study, modify, and distribute their code. This model of software development enables users to adopt and adapt novel computational techniques rapidly and widens their availability. It also promotes collaboration and flexibility. While some of the software packages used in systems biology are available by private license, many software packages are open source, which has enabled the field to develop rapidly. Assembling a community around a specific computational challenge has proven exceptionally powerful. For example, open source, open community efforts such as the github framework connect communities and enable people to build software solutions together. A fascinating concept emerging at the moment is the joint packaging of large-scale datasets and analysis code used in a specific project to ensure full reproducibility by the community.

TABLE 33.1 FAIR principles.

Principle	Definition	Requirements
Findable	Metadata and data should be easy to find for both humans and computers	F1. (Meta)data are assigned a globally unique and persistent identifier F2. Data are described with rich metadata (defined by R1 below) F3. Metadata clearly and explicitly include the identifier of the data they describe F4. (Meta)data are registered or indexed in a searchable resource
Accessible	Processes should be in place for data access, possibly including authentication and authorization	A1. (Meta)data are retrievable by their identifier using a standardized communications protocol A1.1 The protocol is open, free, and universally implementable A1.2 The protocol allows for an authentication and authorization procedure, where necessary A2. Metadata are accessible, even when the data are no longer available
Interoperable	Different datasets need to be integrated and need to interoperate with applications or workflows for analysis, storage, and processing. Use of formal, accessible, shared, and broadly applicable language for knowledge representation is necessary	I1. (Meta)data use a formal, accessible, shared, and broadly applicable language for knowledge representation I2. (Meta)data use vocabularies that follow FAIR principles I3. (Meta)data include qualified references to other (meta)data
Reusable	Metadata and data should be richly described with a plurality of accurate and relevant attributes so that they can be replicated and/or combined in different settings. Conditions and language for data release and use should be clear, accessible and transparent including the provenance leading to the source of the data	R1. Meta(data) are richly described with a plurality of accurate and relevant attributes R1.1. (Meta)data are released with a clear and accessible data usage license R1.2. (Meta)data are associated with detailed provenance R1.3. (Meta)data meet domain-relevant community standards

Means of interrogating biological systems

Even with advances in acquiring and analyzing "big data," one of the main goals of systems biology is to understand the intricacies and interactions of various components of biologic systems. Within the kidney, this requires the use of evolving technologies to probe the flow of information from genes to gene transcripts to proteins and ultimately to metabolites within and across cell types. Systems biology typically analyzes data from various high-throughput technologies, often called "-omics" data, each of which provides the ability to interrogate the genome, transcriptome, proteome, and metabolome [19]. While initial iterations of these technologies were only able to generate organ level data, some technologies now have the ability to provide cell-specific readouts that preserve spatial context. This is increasingly valuable to understanding molecular processes in different cell types based on their anatomical arrangement in the kidney. A sample of the various technologies available, and as appropriate, related computational analytical approaches are described below.

DNA sequencing or the genome

Perhaps one of the earliest uses of systems biology approaches began with the analysis of the human genome. DNA sequencing progressed rapidly from sequencing single genes to the entire genome. This led to Genome Wide Association Studies (GWAS), which explored the relationship between single-nucleotide polymorphisms (SNPs) in the genome, and phenotypes of interest. While initial GWAS studies could only examine 100,000 SNPs at a time [20], the technology and computational methods rapidly improved. With improved chips and the ability to impute other genetic variants based on the individual's haplotype, which can be inferred from publically available data, millions of SNPs can now be examined concurrently [21–23]. The ability to probe the genome was further enhanced with the advent of whole exome sequencing (WES) which involves sequencing all the exons, or transcribed portions of the DNA, base pair by base pair.

Gene expression profiling or transcriptomics

Measurements of the transcriptome involves obtaining a read-out of the relative expression levels of messenger RNA (mRNA) in cells. Initial technologies were based on microarray chips where mRNA isolated from samples was hybridized to corresponding embedded probes, which provided a way to measure mRNA abundance and hence expression level. Since the development of parallel sequencing, RNA sequencing (RNAseq) has now become more common and has several advantages over microarrays [24, 25]. First, RNAseq can measure the entire transcriptome compared to microarrays which were limited by the probes on each chip. Second, RNAseq has greater dynamic range compared to microarrays. More abundant mRNA could saturate probes on the microarray chip and the increased sensitivity of RNAseq improves assessment of low-abundance transcripts [26, 27].

One particular challenge in the earlier days of transcriptional sequencing was differentiating variable transcriptional expression across more than 20 different kidney cell types. One solution for deconvolution involved microdissecting kidney tissue into glomerular and tubulointerstitial compartments prior to sequencing [28–30]. Regardless, even within the glomerular and tubulointerstitial compartments, multiple cell types were present and the expression level of any individual gene represented a weighted average of expression from each cell type. Other computational approaches to deconvolute the cell-specific signal have been used with some success [31].

Computational approaches can be used to extract biologically meaningful patterns from transcriptome data. Enrichment analysis is a computational method to identify overrepresented molecular pathways in a complex dataset. This involves superimposing data on prior knowledge of biological pathways, often using ontologies. Gene Set Enrichment Analysis (GSEA) determines if predefined groups of genes that represent biological pathways are overrepresented in transcriptomic data and associated with a phenotype [32]. The method is broadly applicable and is one of the most commonly used methods in systems biology. While GSEA is a valuable agnostic means to interrogate a dataset, a pathway activation score allows investigators to further investigate specific pathways of interest. This approach quantifies the activation of a predefined biologic pathway across samples or individuals by summarizing the activation of multiple components in a pathway into a single metric [33].

Single-cell and single-nuclear RNA sequencing

Perhaps no other emerging technologies hold the same promise for nephrology as single-cell (scRNAseq) and single-nuclear RNA sequencing (snRNAseq) [34, 35]. ScRNAseq generates gene expression profiles of mRNA from

an individual cell. SnRNAseq is a complimentary approach to scRNAseq and measures a cell-specific profile of the mRNA expression from individual nuclei. Given the different isolation procedures and sources of RNA analyzed, these approaches provide complimentary strategies that can be adapted to tissue sources and questions [36]. There are various platforms and protocols to generate scRNAseq data. All protocols involve gently dissociating cells in the sample to then generate a cell-specific readout of the gene expression profile. These data enable the isolation of cell-specific gene expression responses in heterogeneous cell populations, which makes these technologies particularly appealing for interrogating kidney tissue and disease, given the numerous functionally differentiated cells in the kidney. After normalization and scaling, the high-dimensional data representing transcript reads from thousands of individual cells is projected onto two dimensions using unsupervised clustering algorithms and results can be displayed visually**.** Some of the most commonly used methods for clustering are t-distributed stochastic neighborhood embedding (t-SNE) and Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) [37, 38]. Clusters identified via t-SNE or UMAP can represent either various cell types or states, and require careful annotation using established cell type-specific markers [39, 40].

Initial scRNAseq studies on kidney tissue have identified novel cell clusters that could not be annotated as a known cell type within the kidney [41, 42]. Further investigations are needed to determine if these clusters actually represent previously unknown cell types in the kidney or if they represent different cell states. Moreover, the nephrology field will need to reach a consensus regarding an appropriate level of evidence to designate a novel cell type. However, the identification of novel cell clusters reveals the potential utility of scRNAseq for remaining questions in regenerative nephrology, especially as pluripotent cells (such as stem cells) transition from one cell type to another. Trajectory analysis is another computational approach that has inherent applicability to regenerative nephrology; it sorts individual cells along a timeline or spatial location into an order based on their individual transcriptional profile. These approaches are poised to make significant advances in our understanding cellular changes in the developing, aging, and injured kidney. While information from scRNAseq and snRNAseq will undoubtedly contribute to scientific advances, these technologies fail to preserve spatial information, which will require novel approaches.

Proteomics

Proteomics refers to the measurement of the proteome, or the set of proteins, present within a cell [43]. The proteome can now be measured within kidney tissue itself, urine, or plasma, and each of these may contribute toward a better understanding of kidney disease. The proteome could be considered a functional readout of biologic pathways after considering genetic variants, transcriptional and translational regulation. As such, the proteome is highly dynamic but does capture a more proximal component of disease pathology. There are various technologies to measure the proteome. For instance, mass spectrometry (MS) separates proteins and metabolites based on their mass-to-charge ratio and has been one of the most commonly used techniques [44]. By leveraging the power of other technologies, such as tissue-based microarray immunohistochemistry, it is now possible to obtain near-single-cell resolution of the proteome [45]. Temporal variations and rapid turnover of proteins require careful consideration of the state of the organism, such as the amount of proteinuria, kidney function, time of day, and fasting. All of these factors potentially cause rapid alterations in post-translational modifications of the proteome under study [43].

Metabolomics

Metabolomics refers to the measure of the metabolome or all small molecules measured within a cell at a given time [43]. The metabolome is often considered the ultimate functional product from cellular processes and reflects the sum total effect of gene expression, transcriptional, and translational regulation. The metabolome can be measured in urine, plasma or kidney tissue itself. Recent technology advantages now also consider the spatial context of metabolome differences in kidney tissue [46–48]. Metabolomic analysis has several similarities to proteomic analysis in that the results are typically associative and rapid changes in the metabolite state of a tissue warrant careful consideration to the state of the organism and sample acquisition.

Novel means of capturing histologic and pathologic data

A nephropathologist's visual assessment of kidney tissue is the gold standard for establishing diagnosis of many kidney diseases. While standard pathologic assessment is undoubtedly essential for the diagnosis of patients, alternate means of capturing histologic and pathologic data are valuable in systems biology. Several investigators

have shown that morphometric analysis of kidney tissue by obtaining precise measurements of specific structures and descriptor-based approaches have utility when integrating histopathologic data with other data [49, 50]. Morphometric analysis has been associated with changes in proteinuria in Diabetic Kidney Disease (DKD) [51, 52]. Others have shown that a descriptor-based approach to describe pathologic changes can add a rich layer of detail and granularity to pathologic data, which is reproducible and can be used in data integration [53–55].

The power and potential of data integration

While analysis of data from one omics platform greatly improves understanding of kidney health, development and disease, it only provides a single snapshot of a dynamic process as biologic information is fed through genes, transcripts, proteins, and ultimately metabolites. Integration of data types from various omics platforms holds great potential to generate a "live feed" of a dynamic biologic system instead of a single snap shot of one step in cellular processes. As increasing numbers of patient cohorts now have omics data available and computational techniques steadily advance, the calls for new and improved data integration options are becoming more urgent. This is still an evolving area in systems biology and novel computational approaches are needed to augment the potential of combining the complementary strengths of various data types, especially as some cohorts now have collated data from multiple omics technologies for individual participants. For example, bioinformaticists developed a methodology known as iCluster, which was a machine learning approach that linked differences in gene expression and gene copy number to identify novel subtypes of breast and lung cancer [56]. Recent advances in neural networks, such as Self Organizing Maps, also hold great potential to integrate disparate data types [57–59].

Application of systems biology in kidney health, development, and disease

While systems biology is an evolving and changing field, the application of systems biology approaches has provided novel insights in several areas of nephrology.

Kidney health

Systems biology approaches have enhanced our understanding of kidney health, particularly when understanding the variety of cell types within the kidney. The first scRNAseq studies were done using healthy mouse kidney tissue and successfully identified 16 cell types and noted sex-specific differences in transcriptional expression such as the expression of the prolactin receptor within the female proximal tubule [41, 60]. Moreover, one study identified a novel cell cluster, which expressed cellular markers typically seen both in intercalated and principal cells [41]. Supporting evidence from trajectory analysis suggested that this cluster may represent a transitional state between intercalated and principal cells, although further work is needed to confirm if this cluster truly represents a novel cell type.

While it can be challenging to obtain specimens from individuals who are truly healthy, tumor nephrectomy specimens and deceased donors provide an opportunity to interrogate relatively disease-free tissue. SnRNAseq of kidney tissue of tumor nephrectomies and deceased kidney donors that did not have CKD by estimated glomerular filtration rate (eGFR) and had minimal chronic damage on histologic analysis highlighted the variability present in disease-free human kidney tissue [42]. Here again, novel clusters were identified that could represent transitional cell states within the proximal tubule and intercalated cells, although further work is needed.

Proteomic analysis of the extracellular matrix (ECM) of laser captured microdissected glomeruli from kidney biopsies of deceased donors demonstrated the surprising variety of proteins present. To date, more than 150 proteins have been described, although only 55 of these proteins were independently validated in separate cohorts [61, 62].

Kidney development

Trajectory analysis of scRNAseq data has yielded useful insights in kidney development. ScRNAseq of the human fetal kidney identified progenitor, intermediate and mature cell types, and pseudotemporal trajectory analyses identified three developmental trajectories, including the stroma, collecting duct, and the nephron [63]. Others have completed trajectory analysis of podocyte development, which is a terminal cell type that is unable to regenerate after injury and hence is ripe for investigation into novel regenerative techniques [64]. Kidney organoids are a promising

means to investigate podocyte-specific therapies. Podocytes derived from organoids overwhelmingly recapitulate development of human podocytes, although transcriptional differences are noted late in podocyte development [65]. Of note, many of the transcriptional alterations seen in podocytes derived from organoids can be corrected if they are vascularized during the course of their development, which suggests a potential direction to improve model systems to study podocyte injury and repair [60].

Chronic kidney disease

Chronic kidney disease (CKD) is one of the most common syndromes that nephrologists treat, but apart from blood pressure control and renin-angiotensin system blockade, therapeutic options are limited, as our ability to classify patients based on biologic mechanisms and subsequently prognosticate and treat is limited. Thoughtful application of systems biology approaches to CKD has yielded multiple useful findings in all of these areas.

Gene expression profiling of the tubulointerstitial compartment of more than 200 kidney biopsies from patients with various causes of CKD identified gene transcripts that were differentially expressed between patients with and without progressive CKD. Integrating the results of the genes most strongly associated with eGFR with measurements of the urinary proteome helped to identify urinary epidermal growth factor (uEGF) as a prognostic marker for progression of CKD [66]. Transcriptomic analysis of kidney tissue and publicly available data sources determined that uEGF was a kidney-specific transcript, which increased its prognostic utility. Confirmation in cohort studies demonstrated that uEGF provided additional information compared to urinary protein measurements and eGFR. While mechanistic studies identified EGF decades prior as an important component of tubular damage [67], it was a systems biology approach that ultimately determined that it also had ideal features to serve as a biomarker of CKD progression. It has since been validated in more than 10,000 patients [68, 69].

Clinicians have recognized that CKD runs in families, although the inheritance pattern is clearly multigenic and complex, and heritability estimates hover around 30% [70]. GWAS studies of large cohorts of patients with CKD have identified hundreds of SNPs that are associated with eGFR, albuminuria, and CKD [71]. As cohort sizes have progressively increased, the number of identified variants continues to expand [70, 72–83]. The initial identification of these SNPs requires extensive future investigations to understand if each SNP is truly causal, including fine mapping to identify the actual causal variant, polygenic gene score development, tissue enrichment, and validation in animal models [71]. Combining the results from GWAS with transcriptome data from bulk tissue has winnowed the field of potentially causal variants by determining if the gene of interest is expressed in kidney tissue and the kidney cell type that may be impacted when integrated with scRNAseq data [84].

Others have used systems biology approaches to help elucidate mechanisms of CKD progression. GSEA of the transcriptome from microdissected tubulointerstitial compartments in kidney biopsies demonstrated that fatty acid oxidation was suppressed in samples with CKD [85]. This observation prompted subsequent experiments using mouse models to show that defective fatty acid oxidation in proximal tubular cells promoted fibrosis. Enhancing expression of key regulators in fatty acid oxidation with fenofibrate resulted in decreased expression of apoptotic markers and reduced fibrosis, which has been shown by other investigators [86, 87]. While fenofibrate has yet to be investigated prospectively as a means of slowing CKD progression, posthoc analysis of ACCORD showed that it was associated with decreased loss of eGFR [88].

These investigations highlight that systems biology approaches have the potential to dissect complex and multifaceted syndromes into categories that would be amenable to therapeutic intervention, whether by appropriately identifying patients at high risk of progression, identifying mechanisms that may be drug targets or identifying patients who have underlying genetic causes of CKD.

Diabetic kidney disease

DKD is the most common cause of end-stage kidney disease (ESKD) in the United States, and many patients experience inexorable decline of kidney function despite appropriate medical management. Systems biology approaches may be particularly valuable for studying underlying mechanisms in DKD. Metabolomics approaches have been especially fruitful for studying DKD since it is essentially a disease of dysregulated metabolism.

DKD was perhaps one of the first kidney diseases where morphometric changes were studied [49, 50, 89]. While morphometric alterations, such as increased glomerular basement membrane thickness and interstitial fibrosis are associated with increased loss of kidney function, it was not clear which molecular pathways were involved in these earlier morphometric changes [51]. Weighted gene coexpression analysis (WGCNA), which is a computational approach that identifies groups of coexpressed genes and associates them with various phenotypes, was used

to integrate microdissected tubulointerstitial data from patients with early stage DM and morphometric alterations [52]. Transcripts involved in cell-to-cell matrix interaction, migration, and inflammation were significantly associated with interstitial fibrosis. Importantly, this group of coexpressed genes was associated with increases in albuminuria and loss of GFR 10 years after the initial kidney biopsy.

Metabolomic approaches have been especially valuable to better understand DKD. Integration of transcriptomics, mass spectrometry-based metabolomics, and metabolic flux analysis highlights that changes in energy metabolism in target organs is tissue-specific in mouse models. Expression of enzymes for glycolysis, beta-oxidation, and the tricarboxycylic acid (TCA) cycle were all increased in the diabetic mouse kidney, with corresponding increased flux through all the relevant pathways [90]. Metabolic interrogation of the lipidome has also shown that there is a progressive alteration of the lipid profile as CKD progresses [91]. Moreover, increased abundance of polyunsaturated triacylglycerols and lower abundance of short chain fatty acids were associated with progression of DKD.

Measurement of urinary metabolites has highlighted multiple differences of the urinary metabolome in those with DKD compared to controls, of which 13 metabolites replicated in a separate cohort [92]. Notably, many of these metabolites were substrates of organic anion transporters 1 and 3, and further classification demonstrated that 10 of these metabolites were produced exclusively from mitochondria. In subsequent analysis, four of these metabolites were associated with future loss of kidney function after adjustment for clinical variables [93].

Transcriptome data from patients with DKD showed that the JAK–STAT pathway was highly upregulated in patients with progressive DKD and could be a potential therapeutic target [94]. This result helped spur a Phase 2 clinical trial of Baricitinib, a JAK inhibitor, which decreased albuminuria [95]. The trial made use of target engagement markers that had been identified by a systems biology approach and demonstrated a decrease in inflammatory biomarkers.

Systems biology approaches have immense potential to better understand DKD. As computational approaches continue to improve, we anticipate that systems biology approaches will be able to identify potential therapeutic targets and biomarkers for target engagement and prognostication.

Glomerular diseases

Glomerular diseases are far less frequent causes of ESKD than diabetes, but they are dangerous conditions that can result in rapid loss of kidney function and have a high symptom burden. While glomerular diseases are ultimately diagnosed by histopathologic assessment, it is widely accepted that patients with the same pathologic diagnosis can have varying underlying mechanistic processes. Unraveling these mechanistic processes is of utmost importance, as therapies for glomerular diseases are highly toxic and only half of the patients have complete responses to first-line therapies.

The diagnostic workup and management of patients with what was previously called idiopathic membranous nephropathy were revolutionized when the proteome was measured in the kidney tissue and serum of patients with idiopathic membranous nephropathy and led to the identification of PLA2R as the main antigen in idiopathic membranous nephropathy [96]. After genetic variants in the corresponding gene were identified, PLA2R was considered as pathogenic for membranous nephropathy [97]. Measurements of Anti-PLA2R are now used to diagnose patients with Anti-PLA2R-associated membranous nephropathy and monitor response to treatment [98, 99]. Tissue proteomic technologies are leading to exponential growth in the identification of additional antigens in membranous nephropathy, which may further improve diagnosis and management for these patients [100, 101].

Focal Segmental glomerulosclerosis (FSGS) is a pathologic diagnosis which could more correctly be described as a syndrome that is recognized by the presence of segmentally sclerosed glomeruli. While there are multiple initial insults that start the pathologic process that culminates in a segmentally sclerosed glomerulus, a unifying theme is podocyte loss [102]. However, FSGS has not been subcategorized by mechanistic pathways, but rather by a pathologist's visual assessment of the appearance of the glomeruli [27]. This visual assessment is associated with prognosis and response to therapy, but the accuracy is imperfect [103]. For example, tip variant FSGS is typically steroid-sensitive and has an excellent prognosis, although there are descriptions of steroid resistant tip variant FSGS [104, 105]. Unsupervised clustering of the tubulointerstitial transcriptome of 123 patients with minimal change disease and FSGS identified a cluster of patients with worse prognosis and higher levels of tumor necrosis factor activity. Interestingly, this cluster of patients included those with FSGS as well as those diagnosed with minimal change disease [106].

Proteomic analysis of the extracellular matrix of light-dissected glomeruli has also elucidated the variability and pathogenesis of FSGS. Mass spectrometry of the extracellular matrix in glomeruli with segmental glomerular sclerosis demonstrated that a select handful of proteases, Cathepsin C and annexin A3, were significantly more abundant

in glomeruli with a collapsing lesion [107]. Transcriptome analysis of glomeruli demonstrated that these genes were also over-expressed. Integrating the cell-specific profile of glomerular endothelial cells from scRNAseq data also helped to identify a novel marker for progression for patients with FSGS [108]. Based on the glomerular endothelial cell-specific genes, patients with FSGS were clustered into two groups who had varying prognosis. One of these groups can be identified by higher expression levels of alpha-2 microglobulin transcripts, which is associated with decreased likelihood of remission.

Lupus nephritis (LN) is one of the most severe manifestations of lupus and is associated with high morbidity and mortality. Class III and Class IV LN, a more aggressive subtype of the disease, can commonly fail to respond to therapy, and many of the treatment options carry substantial toxicity, especially for women of childbearing age [109–111]. scRNAseq of patients with LN demonstrated an impressively diverse inflammatory cell infiltrate in various states of activation [5, 112]. Careful profiling of the immune cell infiltrate was then leveraged and integrated with urine proteomic findings, which showed that the urinary proteome correlated with the number of kidney infiltrating CD8 + cells in six patients with scRNAseq [113]. While further work is needed to determine if the urinary proteome of patients with LN can be used to improve prognostication, if these data do highlight the power of integrating multiple omic technologies.

Acute kidney injury

Acute kidney injury (AKI) is a syndrome recognizable by increases in serum creatinine and decreases in urinary output. While episodes of AKI in humans have various causes, including toxic insults, hypotension, and sepsis, one of the most commonly used animal models to understand mechanisms of AKI is an ischemia–reperfusion injury model (IRI), which causes profound acute tubular injury (ATI), one of the most common histologic lesions associated with AKI. As human kidney tissue is not commonly obtained during episodes of AKI for clinical purposes, most systems biology approaches have been applied to data from animal models. The study of AKI is of particular interest in regenerative nephrology as a substantial percentage of patients fail to completely recover from AKI and instead transition to CKD or have further progression of their CKD [114]. While multiple causal biologic pathways have been identified from mechanistic studies, systems biology approaches can prioritize which pathways are most active and map changes in cellular state during AKI, which is a highly dynamic process.

Molecular signatures generated from sequential RNAseq studies of mouse kidneys before and after IRI highlighted the temporal trajectory of biological pathways that are activated after an episode of IRI. Hours after an IRI event, the MAPK signaling pathway, apoptosis genes, and growth arrest pathways are activated, while genes regulating cell death were upregulated for weeks after the initial injury. Genes regulating the adaptive immune response were upregulated months after the initial injury [115]. Further investigations with snRNAseq after IRI in mice identified subpopulations of proximal tubule epithelial cells, including healthy S1, S2, and S3 segments and corresponding damaged segments [116]. Most importantly, one cluster seemed to represent repairing proximal tubular cells as the transcriptional landscape of these cells was enriched for the mitotic cell cycle. In contrast, about 20% of tubular cells seemed to fail to repair as indicated by a proinflammatory molecular signature and decreased expression of terminal differentiation markers. These findings are consistent with mechanistic studies which showed that a subpopulation of tubular cells fail to repair after an AKI event and are atrophied [117].

Human data are limited in the setting of AKI, but kidney transplantation closely mimics IRI and protocol kidney biopsies are commonly obtained on transplant patients, although the immune response after kidney transplantation is likely to differ compared to IRI. RNAseq of protocol kidney biopsies before and after transplantation demonstrated that there are multiple conserved molecular elements after kidney transplantation and murine IRI, although a percentage of patients "failed to repair" [118]. These patients were more likely to have decreased expression of genes involved in mitochondrial homeostasis and increased expression of genes involved in adaptive immunity and fibrosis.

The Kidney Precision Medicine Project (KPMP) has obtained biopsies from patients with AKI, and these samples undergo careful molecular and pathologic interrogation [4]. These data are publically available, and it is anticipated that integration of these data will yield information that will ultimately improve patient care, by either identification of noninvasive biomarkers that facilitate diagnosis or selection of patients amenable to treatment.

Challenges

Systems biology has improved our understanding and knowledge of virtually every form of kidney disease, and in some cases, has drastically improved clinical care. Despite these advances, challenges remain. Perhaps the largest barrier to systems biology research is a dearth of patient specimens that are available for interrogation, especially

kidney tissue. KPMP is the most ambitious attempt, as of now, to obtain and make publically available comprehensive molecular analyses of human kidney tissue from patients with AKI and CKD.

While technologies that provide high-throughput omics data are currently costly, it is expected that with further technological advances costs will decrease drastically, similar to what was observed with genome sequencing technologies. Concerns remain if these costly technologies can be used for patient care. While a multiomics interrogation of each individual patient is unlikely to be feasible in the foreseeable future, intensive analysis of research participants has yielded assays that can be used in direct patient care, such as uEGF and anti-PLA2R. Thus, the initial research investment may be costly, but that does not preclude the development of an affordable assay that can be used for patient diagnosis, prognosis, and treatment.

Additionally, the development of single-cell sequencing technologies was a major step forward for the use of systems biology approaches in kidney disease. While these technologies are powerful and ideal for the kidney, they fail to preserve spatial information. Proper spatial arrangement of cell types is crucial for proper kidney functioning and the interplay between physically adjacent cells is likely a crucial component of the pathogenesis of multiple diseases. The KPMP is incorporating novel technologies that are able to analyze molecular data while preserving the spatial arrangement, which will undoubtedly fulfill an important gap in our understanding of kidney disease [119, 120].

Future directions and conclusions

Given the complexity of both kidney physiology and diseases, systems biology approaches are uniquely suited to help unravel the complexity of kidney disease. Integration of computational methods with multiscalar data comprising molecular, tissue, and clinical parameters could unravel the multiple genes, proteins, and molecular mechanisms that drive key steps. Research efforts, to date, have shown that detailed molecular characterization of kidney diseases helps to decipher underlying pathogenesis of kidney diseases that could lead to more reliable biomarkers to aid clinical decisions, personalize patient care and identify potential therapeutic targets. Systems biology is uniquely suited to unravel some of the scientific challenges that arise when studying regenerative nephrology. It has already elucidated transcriptional changes that occur during kidney development and identified potential adjustments to kidney organoid systems, which will improve their utility as a model system. Systems biology has meaningfully impacted how patients with membranous nephropathy are diagnosed, treated, and monitored and also has added a valuable urinary biomarker to better prognosticate patients with CKD.

We fully anticipate that with the continued improvement of computational approaches and molecular interrogation technologies, the use of systems biology approaches will explode in the coming years. In turn, this will prompt novel discoveries that ultimately improve patient care and management.

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CHAPTER

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Overview of ethical concerns raised by kidney organoids

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Chronic kidney disease (CKD) is currently known to affect 11%–13% [1] of the population worldwide. In the United States alone, it is estimated that approximately 37 million adults have CKD, yet most are undiagnosed [2]. CKD is also expected to become the fifth leading cause of death by 2040 [3]. A significant proportion of patients with CKD will progress to end-stage renal disease (ESRD), and it is an independent risk factor for cardiovascular disease [4].

Therapeutic options for patients with CKD are often limited to dialysis and organ transplantation [5]. In 2016, nearly 125,000 people in the United States started treatment for ESRD, and more than 726,000 were on dialysis or were living with a kidney transplant [6]. The latter statistic represents two in every 1000 people. Renal replacement therapy (RRT) with dialysis and/or transplantation represents major costs for healthcare systems [7]. Moreover, those costs increase exponentially with each CKD stage progression [8–10]. In recent years, there has been an increase in the number of patients utilizing home dialysis; however, for patients undergoing facility-based hemodialysis, three times a week remains the standard of care for the majority of patients [11]. The detriments of ESRD and its treatment on patients are not only physiological and financial. ESRD is also associated with increased psychological distress and diminished quality of life for patients and family caregivers [12, 13].

Regenerative nephrology offers new possibilities and hope for curing chronic kidney disease. Current research and advancement in biotechnologies can provide the possibility in the near future to grow synthetic kidney tissues in the laboratory. These kidney organoids [14], i.e., aggregation of cells that differentiate and self-assemble in ways similar to cells of a developing kidney, are created from pluripotent human embryonic stems cells (hESCs) or induced pluripotent stem cells (iPSCs). They can be used by pharmaceutical companies in drug development and toxicology testing to run what would be akin to in vitro clinical trials. These trials would be more cost effective than traditional clinical trials. They may also increase the overall probability for drug approval due to the ability to run trials without many research participants. Moreover, organoids may eventually be used to repair dysfunctional organs or serve as replacement organs, thereby solving the global organ shortage for transplant. In particular, kidney organoids that are developed through iPSCs are derived from cells that come from the patient himself or herself; therefore, they could provide immuno-compatible tissues and provide personalized therapy. Researchers are also considering how to generate kidneys without mutations associated with congenital disorders through the use of gene-editing tools, such as CRISPR.

Despite those extraordinary benefits, however, regenerative nephrology may raise some ethical concerns in relation to (1) the use of human pluripotent stem cells, (2) animal experiments, in particular with respect to using host chimera, and finally (3) with regard to patient consent and therapeutic misconception. We will address these concerns in the following sections.

Kidney organoids from human pluripotent stem cells (eHSCs and iPSCs)

Creating an artificial kidney or a kidney organoid is a tremendous challenge due to the anatomical complexity of this particular organ. Kidneys contain many cell types that interact in a complex three-dimensional architecture. Because of these anatomical complications, the kidney is believed to be one of the hardest organs to reproduce [15].

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However, with the great progress made in our understanding of the kidney's embryonic development, researchers have been able to generate the first kidney's organoids 5 years ago [16]. While kidney organoids carry a lot of promise, there is still a long way to go, as they only represent an immature version of an adult kidney. For example, current kidney organoids that have been created still lack vascularization as observed in vivo. Moreover, there is still variability, scaling, and size issues that researchers must overcome before being able to apply the science to any potential clinical breakthrough [17].

Organoids derived from hESCs first raised ethical concerns in the 1990s, since the procurement of embryonic stem cells was tied to public controversy surrounding abortion. Typically, human embryonic stems cells are derived from embryos discarded at IVF clinics, which are used as the starting material from which hESCs will be collected and expanded. The ethical debate regarding using hESCs for scientific and medical research has preoccupied the public for more than three decades. In particular, religious communities have raised concerns regarding the production of new stem cell lines, involving the further destruction of embryos.

During his presidency, President George W. Bush vetoed federal funding for hESC lines created after 2001. However, the NIH was allowed to fund hESC lines already developed or in development prior to the enactment of Bush's executive order [18]. Private funding could be also used—and still is used—for research on newly created human embryo-derived cell lines. President Barack Obama removed Bush's restriction of federal funding for research for lines derived after 2001 by an executive order, "Removing Barriers to Responsible Scientific Research Involving Human Stem Cells" [19]. During the course of his presidency, the NIH approved more than 300 different new hESC lines derived from supernumerary frozen embryos, based on the assumption that "the research has scientific merit and the embryos were donated after proper informed consent was obtained from the donor(s)" [20]. Whereas the controversy about the status of the embryo remains, most researches currently use hESC lines that have already been derived and can be kept propagating, which makes the destruction of new embryos unnecessary.

Some nevertheless disapprove of research using hESCs due to the concept of the "fruit of the poisonous tree," which means that because embryonic stem cells were procured in an immoral way, then anything gained from the research is tainted as well. However, even among those ethicists who deem abortion to be immoral, most nevertheless consider knowledge gained from the research to be morally beneficial if not neutral. Moreover, now that hESCs can be kept propagating, those ethicists do not see the value in relying on the "fruit of the poisonous tree" concept to avoid any potential slippery slope, where more hESCs may need to be procured through carrying out abortions.

Creating organoids through iPSCs has also been considered an elegant way to avoid the controversy raised by hESC lines, as they do not require the destruction of embryos. Rather, iPSCs are made through the genetic reprogramming of somatic cells [21]. iPSCs are routinely generated by the forced expression of several transcription factors in somatic cells. The cells are collected directly from the patient, most frequently by a small skin biopsy, and can differentiate into all cell types of the body just like hESCs. iPSC holds great promise for personalized medicine as they carry identical genetics as the host, since they are derived from him or her.

Kidney organoids, animal experiments, chimera, and xenotransplantation

There has been a lot in the ethical literature regarding the moral implications of the development of cerebral organoids or neuroloids [22], which are three-dimensional neural tissues cultured in vitro. The advances in neural development and increased ability to model diverse brain regions have also catalyzed media attention and public fear about the possibility, as science progresses, that mini-brain-networks [23] could exhibit moral features such as sentience and consciousness [24]. Some have even referenced the famous "brains in a vat" thought experiment formulated by Hilary Putnam in 1981 [25]. These questions, and whether those fears are more myth than reality, however, fall out of the scope of this paper, since cerebral organoids and other organoids have very different moral implications due to the potential for consciousness. Moreover, kidney organoids do not exhibit any of the *moral status signifying features*, i.e., as those that are identified with independent life such as a heartbeat, which can trigger reactions of moral repugnance. This "yuck factor" [26] is not an issue with kidney organoid development and society has welcomed it with great excitement.

The main issue of concern regarding kidney organoids revolves around the ethics of animal experimentation. For example, recent research has been in attempting to generate rat/mouse interspecies chimeric kidneys that can function in vivo [27, 28]. In the near future, it is projected that porcine fetuses will be used as a bioreactor via in vivo methods to create regenerated kidneys with sizes similar to those of humans.

Within the realm of proper methods in animal research, ethicists traditionally use the "3 Rs Approach," whose principles, i.e. Replacement, Reduction, and Refinement, are considered essential for good conduct in animal research.

Originally developed by William Russell and Rex Burch in 1959 [29], it was given with very little attention by the public; however, those principles have been increasingly incorporated into legislation, guidelines, and practice of animal experiments [30–32]. The "3 Rs Approach" stipulates the following: Animal experiments should be "R-eplaced" by alternative techniques when they are available, to avoid unnecessary harm to nonhuman species. The number of animals used in experimental research should be "R-educed" to a minimum. Finally, the "R-efine" principle demands that experiments be carried out in ways that involve as little pain and distress to the subject animals as possible [33].

Personalized kidney organoids, created through iPSCs and gene editing or single-cell sequencing technology [34], may have the potential to reduce the number of animals used in experimentation [35]. Historically, animals have been used as the initial test subjects, before clinical translation to humans. The traditional reliance of biological research on animals has made it challenging to extrapolate results to humans, serving to be a major bottleneck in the drug discovery process. Kidney organoids derived from iPSCs present great potential for disease modeling, drug screening and nephrotoxicity testing and offer new alternatives to animal experiments [36–38]. Kidney organoids derived from iPSCs may actually be a better model for kidney diseases than using animals. In vitro models can replicate phenotypes, allowing one to test toxicity and efficacy. Organoid models can also be directly generated from affected patients without prior knowledge of the specific genes responsible and can, consequently, avoid unnecessary animal genetic manipulation in the first stages of drug discovery process.

One successful use of kidney organoids already has been in examining polycystic kidney diseased (PKD) organoids to study autosomal-dominant polycystic kidney disease (ADPKD), a common genetic present in approximately 5% of patients with total ESRD. Researchers have shared their enthusiasm regarding the possibility of creating "clinical trials in a dish" to "replace animal testing, or to be used to select patient populations or even individual patients most likely to benefit or least likely to be harmed by therapies" [39]. However, the development of organoid technology is still quite new. At the moment, it serves more as a complement than a replacement of animal testing. Moreover, it is unlikely that kidney organoids will fully replace animal testing at all stages of drug development since alternative methods for drug validation are necessary [40]. Those precautionary measures, required before proposing clinical trials in humans, drive the present regulatory framework for FDA drug approval and are unlikely to be changed.

Combining human and animal biomaterial [41] for kidney organoids and xenotransplantation are also important areas of discussion regarding their ethical implications. Scholars have flagged the risk of creating "humanized" mice or immune-deficient mice engrafted with functional human biological systems [42]. However, kidney organoids do not demand the same level of scrutiny as other types of chimera research, where complex in vitro structures of human origin are introduced in the brain or reproductive systems of nonhuman animals, since those factors that symbolize or indicate human consciousness are not present. Therefore, ethical examination remains in the realm of proper conduct for animal research. It does not blur lines between animal research and research on human subjects.

Some worry that chimerism could inadvertently go beyond the localized formation of human organs—in particular, when the interventions are performed at the earliest stages of development with, for example, pig fetuses—which may result in an acutely chimeric animal with an ambiguous moral status [43]. However, the scientific community has already mitigated those concerns by providing ethical guidelines for chimera research to avoid such consequences accidentally [44]. The transfer of human organoids in animal models must be closely monitored to assess the physical and behavioral change that may occur in the host organism [45]. The Ethics and Public Policy Committee of the International Society for Stem Cell Research (ISSCR) [46, 47] provides some recommendations which include the following: (a) establishment of baseline animal data; (b) ongoing data collection during research concerning any deviation from the norms of species-typical animals; (c) the use of small pilot studies to ascertain any welfare changes in modified animals; and (d) ongoing monitoring and reporting to oversight committees authorized to decide the need for protocol changes and the withdrawal of animal subjects [48].

As the possibility of growing human kidneys inside human-pig chimeras is becoming more than mere science fiction via a technique known as interspecies blastocyst complementation, xenotransplantation trials have raised more and more debates. Animal rights advocates, in particular, have raised concerns regarding the possibility of farming human-pig chimeras for the sole purpose of growing and harvesting human organs. We argue, however, that farming human-pig chimeras for their organs is no more ethically concerning farming nonchimeric pigs for food [49]. Some advocates have endorsed taking a precautionary approach to human-pig chimera research because of the concern mentioned above about the possibility that such chimeric animals develop new morally signifying traits [50]. However, kidney organoids are unlikely to change the moral status of a pig chimera in comparison to interventions specifically targeting the central nervous system or the germline. Whereas animal welfare standards need to be carefully monitored, especially when research requires livestock animal, these concerns should be weighed in balance with the priority to advance therapeutic research and how it may be possible to improve living conditions for animals raised in intensive farming systems on factory farms.

Patient consent and therapeutic misconception

Patients with ESRD face a severe shortage of transplant kidneys. The inevitable consequence of such a shortage is a very high mortality rate. Over 126,000 patients are treated every year for ESRD, yet only about 31,000 (25%) are added to the waiting list to receive a kidney from a deceased donor. Among those on the list, only 20,000 (16%) actually end up receiving a transplant kidney [51]. Since most patients in ESRD wait an average of 3.9 years to receive a donor kidney, 35% of transplant candidates die while waiting for organ transplant or have been removed from the waitlist as their health starts to decompensate [52]. According to estimates, 63,000 patients who are not on current waiting list for organ transplant might medically benefit from a transplant, if they were only able to get one [52]. If there were enough kidneys for every patient who needed one, approximately 43,000 lives could be saved every single year.

Because of the benefits that regenerative nephrology can provide to ameliorate the dire situations of those patients with ERSD, there is great enthusiasm for its development. However, it remains unclear whether transplanted kidney organoids will eventually become safe or functional. The immune response to a xenograft is generally more extreme in interspecies transplantation, which may lead ultimately to xenograft rejection or, in severe cases, death of the recipient. Yet, there is some room for hope. Recent studies in mice have addressed safety concerns regarding graft immaturity and maldifferentiation of the kidney organoids derived from iPSCs. The use of modified or transgenic organ donors could also help prevent the recipient's immune system from immediately rejecting the xenograft. Other strategies are being developed as well, such as the tolerance approach, which consists of administering donor's bone marrow cells before transplantation to trick the recipient's immune system into recognizing donor molecules as part of the person himself or herself [53].

Because of those safety concerns, discussions have revolved over what would be the appropriate conditions, once the technology becomes available, for selecting patients for initial clinical trials of solid organ xenotransplantation. Some scholars have suggested that some recurrent kidney diseases that are known to affect 12%–50% patients with CKD to ESRD should be included among the eligible conditions for initial clinical trials of pig kidney transplant. For instance, patients with recurrent focal sclerosing glomerulo-sclerosis (FSGS) or recurrent immunoglobulin A (IgA) nephropathy would fall in that category. Other studies have argued that initial clinical trials of pig kidney transplant should be provided only to patients with ERSD. The rationale is that it would be "ethical to provide pig kidney transplant to selected patients who have a life expectancy shorter than the time it would take for them to obtain a kidney from a deceased human donor," as this would be beneficent without having the patient incurring additional risks to life expectancy [54]. A significant portion of patients who are not currently on a waiting list for organ transplants and who are deemed as those who could benefit clinically from organ transplantation could also be considered potential candidates. The USFDA currently recommends that xenotransplantation should be limited to "patients with serious or life-threatening diseases for whom adequately safe and effective alternative therapies are not available," while limiting patients to those "who have potential for a clinically significant improvement with increased quality of life following the procedure" [55]. Once the technology becomes available, the medical community needs to define the appropriate criteria for xenotransplantation, aligned with ethical standards. Most importantly, therapeutic misconception, in particular during phase I of clinical trials, will need to be addressed to ensure patient informed consent. Whereas the risks/benefits analysis of phase I clinical trials is often conceived by the public as follows: "anything is better than death," we need to remember that initial clinical trials of pig kidney transplant will necessarily entail additional burden on patients and families and could potentially affect end-of-life quality.

Finally, it is worth noting that kidney xenotransplantation may actually decrease the number of ethical dilemmas in actuality [56, 57]. Refusal of dialysis is one of the most common reasons for initiating a clinical ethics consultation. Balancing the benefits of dialysis (life-sustaining) with its major burdens (restrictive and time consuming) involve complex value judgments [58, 59]. The increase in available organs for transplant may decrease the complexities of patient refusal and/or compliance with dialysis.

Conclusion

In a 2015 *Boston Globe* op-ed, psychologist Steven Pinker wrote that "the primary moral goal for today's bioethics can be summarized in a single sentence. Get out of the way" [60]. Praising the advances made by biomedical research and the opportunities they afford, such as increased lifespan, decreased rates of premature death, and curing disease, Pinker offers that the greatest obstacle to enjoying further benefits of biomedical advancement is ethics. The reason is that bioethicists often get mired in the muck procedural detail, stymying ethical researchers from doing their job. He

proposes, "A truly ethical bioethics should not bog down research in red tape, moratoria, or threats of prosecution based on nebulous but sweeping principles such as 'dignity,' 'sacredness,' or 'social justice'" [60].

We agree with Pinker that regenerative nephrology offers new possibilities and hope for curing chronic kidney disease, which will also increase lifespans and decrease rates of premature death. We also believe, along with Pinker that the majority of biomedical researchers are intrinsically motivated to make the world a better place. However, we also recognize that ethical conduct of research and ensuring that the opportunities afforded to people who can potentially benefit from that research should be based on firm ethical principles. Knowledge of potential ethical concerns raised by regenerative nephrology and the understanding of how to respond to them will allow future patients to live longer lives of higher quality.

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CHAPTER

35

Manufacturing challenges and solutions for regenerative medicine technologies

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Introduction: Regenerative medicine manufacturing landscape

There have been numerous reviews that signify the benefits of manufacturing principles applied to regenerative medicine (RM) [1–4]. This chapter will highlight some of the main manufacturing challenges that still exist and propose strategic solutions to address these shortfalls. In essence, manufacturing principles applied to RM are needto scale up the therapies and technologies, standardization of the raw materials used for manufacturing these therapies and technologies, and assurance of widespread access to these technologies by permitting the adoption and distribution at a cost point that enables wide patient access. Applying manufacturing principles to RM from development stage can accelerate the translation process. We will give some examples throughout the chapter that can help speed up the translation of RM therapies and technologies and reduce costs as well. We will also discuss manufacturing of bioengineered complex organs and give some examples of the costs associated with tissue engineered clinical products, although there is still great need for more cost data for these products.

Manufacturing challenges and solutions for regenerative medicine technologies

The ultimate aim of any RM technology is to restore structure and function of damaged tissues or organs. Today, RM products include a broad array of emerging therapeutics including cell therapies (both stem cell therapies and immunotherapies), engineered tissues and organs, gene therapies, cell-based devices, and cell-free implants and materials that are designed to aid in tissue regeneration in vivo [5]. Though the principles of RM are easy to apply to develop technologies which offer a great potential to address unmet clinical needs, only a few have made to clinical trials. Failure of most RM technologies in reaching to clinic can be attributed to significant challenges in manufacturing that either fail the technology to be translated or slows down the process to move the technology from bench to bedside. There are several parameters critical to manufacturing that present challenges. While each of these parameters contributing to the manufacturing process has to be optimized, controlled, regulated, and standardized, the manufacturing process itself has to be robust and sustainable to deliver a quality clinical product (Fig. 35.1). Another significant hurdle is the cost of manufacturing these technologies.

Raw material challenges and solutions

In enabling a RM technology, there are several raw materials that contribute to the manufacturing of the final clinical product, but there are three important starting raw materials that hold the higher place: cells, scaffold, and growth factors. While a technology can use standalone starting material or a combination of two starting materials or all three, these form the first raw materials for manufacturing a RM product. Of these three, cells pose the major challenge depending on type of cell being used for the technology. Based on the cells, RM therapy is grouped into two categories:

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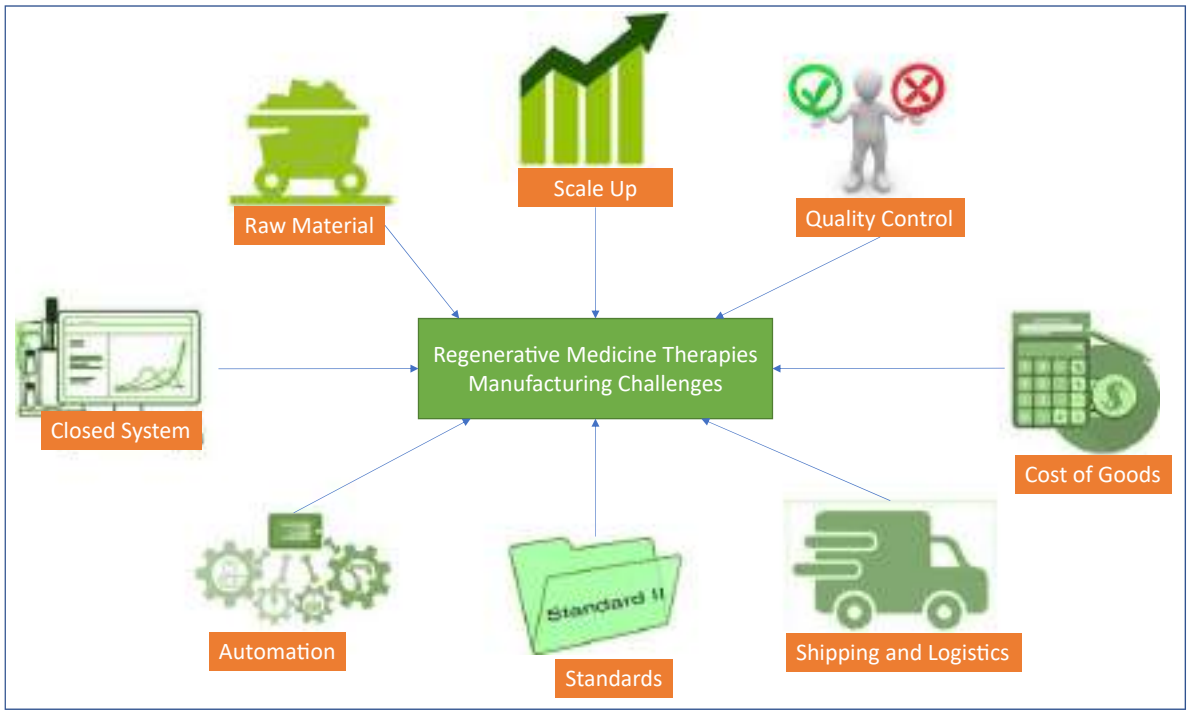

FIG. 35.1 Regenerative medicine manufacturing therapies challenges. Illustrated are highlighted challenges for regenerative medicine manufacturing that are discussed in this chapter.

autologous, in which patient's own cells are harvested, manipulated in ex vivo (either in laboratory, processing center, or clinical setting) and reintroduced back into the same patient, or allogeneic, in which patient receives therapeutic products manufactured using cells from a donor.

Manufacturing a safe quality product starts with the use of validated and qualified raw material. Raw materials are also known as ancillary materials and interested readers are referred to this review which provides more details on the importance of ancillary materials in developing safe cell therapies [6]. While the field of RM therapies is growing rapidly, the guidance that dictates the regulations for cGMP (current Good Manufacturing Practice) manufacturing of the RM clinical product or the raw material used in manufacturing of RM clinical products is still underdeveloped and is still evolving. For instance, most of the guidance documents are adopted from drug or medical device regulations. In spite of evolving guidance, dedicated global regulations are still being developed for the manufacturing of raw materials and qualifying them for use in RM technologies. As a result, no universal standards are available for the manufacturing of GMP or clinical grade raw materials exclusive for RM technologies. The major challenge with raw material is availability of clinical or GMP-grade raw material when transitioning from preclinical to clinical stage. Manufacturers of raw materials, however, qualify by following all applicable GMP guidelines recommended for pharmaceutical products. These guidelines adopted from GMP guidelines for pharmaceutical products provide guidance for manufacturing, testing, and quality assurance. Raw materials manufactured in compliance with all applicable GMP guidelines for pharmaceutical products ensure documented evidence provided as certificate of analysis, certificate of origin, and certificate of compliance for purity, potency, consistency, and stability. Manufacturing under GMP conditions is therefore one of the major quality assurance systems to ensure the safety and high quality of raw materials.

Another challenge is even if the clinical or GMP-grade raw material is available, reliable continuous supply of the material can be a challenge if there is only one vendor in your supply chain. For a smooth transition from bench to bedside, identifying the appropriate raw material and the supplier with a backup supplier or backup raw material early in the game as early as preclinical studies can leverage cost and time. Comparative studies should be performed at an early stage to ensure that the use of required GMP-grade raw materials from the backup vendor or the backup raw material does not alter the final clinical product. Changing the raw materials at a later stage in clinical development often calls for time-consuming comparability studies which adds significant additional costs and time.

Raw materials used during the manufacturing are usually classified under two categories: ancillary or excipient. Ancillary materials are those that come in contact with the cell or tissue product during manufacturing but are not intended to be part of the final product formulation. Excipient materials are inactive ingredient intended to be in the

final product. However, some ancillary materials may be considered excipients if intended to remain in final product. Whether excipient or ancillary, using a raw material that is not animal-derived and manufactured using animal-free components would significantly reduce the qualification and validation efforts. Raw material supplier should be able to provide drug master files (DMF) or regulatory support documents along with extensive stability data if possible. As of now, GMP-grade raw materials should be in compliance with USP chapter 1043 [7], and the upcoming ISO Technical Specification 20399 on Ancillary Materials.

To address some of the challenges with raw materials such as media which is most basic for lots of RM technologies, some groups are developing defined, xeno-free media products to assist with clinical cell manufacturing. These media products do not include any animal products or serum. These media support the expansion of clinically relevant cell types and can be optimized for any manufacturing process. Building such platform technologies as these will be instrumental in reducing variability and ensuring clinical-grade products are incorporated early into a manufacturing process.

Cell expansion challenges and solutions

Most of the RM therapeutic products are cell-dependent. Whether it is cell therapy, gene therapy, or a tissue-engineered product, cells are the crucial part of the final clinical product. Cells are the living component that are sensitive to their environment and a simple manipulation can change their properties. Culturing, expanding, characterizing, and cryopreserving cells are the critical parameters that are dependent on the environment and demand a great deal of development. There is a tremendous gap in the intrinsic understanding of the cells in culture which accounts for variability observed even in tightly controlled environments. On a broad basis, this variation can be attributed to two factors: input material and expansion conditions. Preclinical studies translating to clinical development challenge the scale-up strategies. Scaling up to 10–1000 folds calls for planned approach with check points to validate the manufacturing process. For many cell therapies, production of appropriate cell lines needed for therapies is limited by quality and quantity of the starting material [8].

Quality and quantity of the source from which the cells are isolated play a critical role in determining their potential to make it to final clinical product. This holds true to autologous cell therapies or tissue-engineered constructs that use autologous cells, i.e., where patient's own cells are used to manufacture the final clinical product. Cells isolated from tissue obtained from patient vary significantly based on patient's cellular compositions and physiological states which become a significant challenge from the start of the manufacturing process, as the cells vary not only in initial quality but also in initial yield from patient to patient. In such cases, it is challenging to set a tight specification for product release criteria which is a requirement for clinical products. Regulatory agencies such as the FDA are providing guidance on potency tests for cellular and gene therapy products which propose acceptance criteria for product release [9].

Preclinical studies translating to clinical development usually pose scaling challenges where it is important to understand what strategy to use, scale up or scale out. Scaling to 10–1000 folds calls for planned approach with check points to validate the manufacturing process. Generating these larger batches of therapeutic products from a single source of starting material is difficult to apply to autologous cells. For autologous cell isolating from a small biopsy, expanding to larger numbers while maintaining lower passage number is challenging. Thus, scale-out strategies could be used instead of scale-up or a combination of scale-up and scale-out. In such cases, manufacturing consistencies are difficult to achieve.

In addition, the traditional model of scale-up of a production process, which includes generating bigger batches of therapeutic products from a single source of starting material, does not function well for an autologously sourced production. In cases where source material is less, expanding to an industrial-scale production process must follow a scale-out model in which, to expand in scale rather than producing more cells in each batch, more batches must be made. The variability inherent in the starting materials from each patient will always exist, so the scale-out process may need different control strategies than the scale-up process to achieve manufacturing consistency. In this scenario, methods for defining and measuring consistency may require additional exploration.

Sources from where cells are obtained range from primary fetal cells to various types of fully differentiated cells. These cells can be obtained from in vitro expansion, from a cell line, or can be primary cells isolated from tissues. During some manufacturing processes, cells are preselected based on their phenotypic characteristics. The developmental maturity of cells needed for the therapy vary depending on cell type and therapeutic needs. For some therapeutic applications, immature cells are transplanted which undergo further maturation in vivo, while in some therapeutic applications, cells are matured in vitro [5].

In many cases, only a small number of cells are isolated/extracted which needs further expansion for weeks to reach the number required for manufacturing the therapeutic product. All through this expansion, it is critical that cells maintain their original phenotype, genotype, identity, and function. End of production passage number along with valid cryopreservation studies become essential when dealing with cellular material. The broad range and complexity of cell

sourcing and processing demands the development of appropriate validation assays to ensure the maintenance of cellular characteristics. Though there are almost 80 different cell types that are being used for different cell therapies, not all of these cells have specific markers to identify them such as mesenchymal stromal/stem cells (MSCs) for which data indicate that the current set of surface markers used to identify cells are insufficiently specific to ensure their functional quality [10, 11]. More refined characteristics of cell quality are therefore critically needed [12].

Additional solutions for cell expansion beyond more defined methods for cell characterization include advances in bioreactor technology. These advances will move toward 3D culture and expansion platforms that incorporate scaffolds and extracellular matrix proteins that mimic the cells' native environmental milieu into the growing and expansion of cells. Across this process, cells will need to be monitored noninvasively to ensure that the correct phenotypic profile is maintained across this manufacturing process. These bioreactors will also need to be developed to mature cell seeded tissues and organs which are derived from decellularization methods, 3D bioprinting, or other novel bioengineering strategies.

Quality control and standards challenges and solutions

The quality of clinical product is controlled by instituting a robust quality control system to maintain the consistency of final product during each production run. Quality control testing should be performed on every component at different stages of manufacturing starting from the raw material used, to the final product that is manufactured to ensure the safety, purity, identity, and efficacy of the clinical product being manufactured. Source, origin, and suitability of each raw material play a crucial in producing a safe quality clinical product [13]. In most cases, use of raw material such as consumable and reagents in manufacturing RM product relies mostly on certificate of analysis, certificate of origin, and certificate of compliance; however, there are still some critical raw materials that do not come with appropriate documentation and thus require additional testing to qualify the material to be released for use.

In many RM products, as discussed earlier cells signify the starting material which are qualified by identity and purity testing by studying the expression of cell-specific immune phenotypic markers. But apart from this, it is also essential to check the immunomodulatory function, cytokine profile, and stability of the cells. In-process quality testing during culturing and expansion of the cells become crucial to assess the quality of cells. Other important characteristic that are essential to study include tumorigenicity, toxicity, biodistribution, and genomic stability of cells particularly in cell based therapies. While preclinical studies evaluate the efficacy of the final product, they also help in accessing the genomic stability, tumorigenicity, toxicity, and biodistribution.

Additional solutions regarding quality standards for RM therapeutic products are coming out of groups such as Standards Coordinating Body which is supporting the development of standards and best practices. Current projects underway include characterization of fiber-based scaffolds, characterization of human cells for therapeutic use, and others.

Automation and closed system challenges and solutions

Most important aspect of any manufacturing process is reproducibility and consistency. Manufacturing of RM therapeutic product is a complex process with very stringently controlled steps that involve in-process quality monitoring during the manufacturing process. A minor variation in the manufacturing process can compromise the quality and efficiency of the final product. Manual errors and open systems can introduce variations even in a well-developed process.

Automation with a closed system can help reduce variability while enabling reproducibility and scalability to standardize and simplify the robust manufacturing processes guaranteeing a safe and effective clinical product. Automation is very common in manufacturing world, but automation for manufacturing RM product is an emergent technology. Manual operation in small-scale bioprocesses are more economical in the short term, but pose high risk of variations among different operators, difficulties in transferring to another site for processing, increased possibility of contamination, inconsistent processing, overall leading to increased product variation. Adopting closed systems that are fully automated not only eliminates the operator-related variations but also eliminates the risks of contamination while improving the process capability and consistency.

Automation has been adopted in large-scale production of cell culture in pharmaceutical sector such as large-scale Chinese hamster ovary (CHO) cell culture for pharmaceutical applications [14], but adherent cell culture-based therapies are not readily adopting automation for commercial scaling-up. Automation of adherent bioprocessing techniques is an important aspect of translation to progress the RM therapeutic products to commercial reality [15]. With rapid expansion of cell therapies and gene therapies, soon, need for mega-scale production of cells will be in demand and to successfully meet that demand, automation is the way to go. Managing the risks of product variability and

contamination becomes more crucial when mega-scale production is needed. Automation can be expensive in the short term but the long-term benefits of superior quality product that is manufactured in a closed-automated system with consistent and controlled processes are needed for commercial success.

Currently, disposable closed culture systems are being used for patient-specific cell-therapy bioprocessing such as isolation of hematopoietic stem cell and T cell [16]. Disposable closed systems have the advantage of increased efficiency due to elimination of sanitation steps and reducing the risk for cross-contamination between different manufacturing runs. Potential disadvantages of single-use disposables are the need to have sufficient inventory on hand and also to ensure that there are multiple suppliers.

Automation can be categorized as semi-automation or full automation. Semiautomation is when only a part or a step or subprocess of the complete bioprocess is automated. Automation of in-process monitoring techniques, either as part of a fully automated platform, or as generic automated modules for subprocesses, is useful for reducing the often subjective and time-consuming nature and potential variation of current in-process measurements, which compromise accuracy [17]. Many semiautomated bioreactors are being widely used for large commercial scale production of cells that has remote monitoring and culture maintenance abilities. Robotic processing and real-time monitoring with improvised process control technology can enable robust and reproducible bioprocesses for cell-based therapies. Some of the examples of automation in market used for cell culture include Automation CompacT Cellbase (The Automation Partnership) for static culture which offers low-risk process transfer and regulated production heritage for biologics [e.g., Erythropoietin (EPO)]; Perfusion bioreactor such as PluriX 3D Bioreactor (Pluristem Therapeutics) WAVE Bioreactor system (GE Healthcare); and Suspension bioreactors such as Advanced microscale bioreactor (The Automation Partnership). Advanced microscale bioreactor (Ambr) system (The Automation Partnership) is using the concept to build and develop new technologies that have a high-throughput automated platform comprising up to 48 miniature suspension bioreactors with controlled temperature, pH, dissolved oxygen, and carbon dioxide tension and stirring. This automated platform designed to emulate larger scale commercial suspension bioreactors primarily developed for CHO culture highlights development of cost-effective scaled-down automated systems for use in research and preclinical development to aid bioprocess translation and adoption of automation [17].

Automation is important for overcoming translational bioprocessing issues, as it can improve process capability, remove operator-dependent variation, understand process variation by allowing the automation of complex experimental protocols, and enable scalable culture with controlled cost [18–23]. Automation capable of replicating manual processes will help streamline and standardize manufacturing process of RM therapeutic product and eliminate highly individualized manufacturing strategies for each product in development. A move toward device-centric manufacturing from process-centric manufacturing needs knowledge of variation levels in manual processes along with regulatory requirements to ensure quality product. This is essential to understand what factors need to be considered to design automated next-generation bioprocessing production systems.

Another solution for automation and closed systems is based on modular GMP environments which are now commercially available. One system is from Biospherix, which can assist in creating different manufacturing modules for each process and controlling the conditions to ensure optimal environments for cell manufacturing. These modular GMP environments could, in the future, replace brick-and-mortar GMP facilities and offer up a more flexible and modular designs for GMP manufacturing of regenerative medicine products.

Shipping logistics and storage challenges and solutions

Temperature-controlled containers for traditional pharmaceuticals through to organ transplants and blood products already exist. Transporting the final product from the site of manufacturing to site of use is highly developed in case of pharmaceuticals and devices; however, when it comes to RM therapeutic product, the shipping system is not well developed.

Several factors need to be considered when deciding on a route to deliver a cell therapy product to the clinic. Oldest and most common examples of a cell therapy is transfusion of red blood cells (RBCs) in cases of severe anemia, which relies on refrigerated storage. Donated blood is usually transported to one of several national blood banking facilities where it is processed and then RBCs are preserved in an additive solution to give a shelf-life of up to 42 days [24]. They are subsequently transported to hospitals where their storage and use are closely monitored to manage waste and comply with regulations [25].

Complexity in transporting manufactured RM therapeutic product also arises, as the products manufactured are either personalized products for patient or matched to donor-recipient where traceability is extremely important aspect. End-to-end traceability becomes an essential part of product when patient's own cells are used for manufacturing the therapeutic product. Shipping logistic with traceability has to be carefully planned in case of

personalized product manufacturing which includes transporting patient's tissue or biopsy to the processing or manufacturing center for isolating patient's own cells and manufacturing therapeutic product from those isolated cells followed by transporting the manufactured therapeutic product to clinic for implantation.

Shipping method usually depends on site of manufacturing, site of use, and conditions of preservation. Cryopreservation is one technique that many RM products use to maintain and store for long term, as well as to transport the product at optimum temperature either using dry ice or vapor phase nitrogen. Temperature-control logistic solutions is crucial for transporting materials from the site of harvest to manufacturing facilities and to clinical centers under strictly controlled conditions. Though refrigerated trucks and shipping containers are used for transportation of frozen foods, cellular therapeutic products require a more specialized solution to be maintained at the cryogenic temperature of between −160°F and −240°F to suspend all metabolic processes. Cold chain provider for transporting cellular therapeutic is a rapidly growing market. Shipping at that low temperatures is done using dewar filled with liquid nitrogen boxed in and shipped in freight along with sensors that record real-time temperature, pressure, geolocation, shock, and light exposure. Delicate care in handling and placing the shipment during transport is essential, as orientation of the container matters and has to be kept right-side-up. Some product need shipment with dry-ice and some have to be shipped at ambient temperature. During the shipments, it is paramount that studies are established to understand the impact of additive, media, solution or cryopreservative during transport and stability of the product for the duration of shipment at different temperatures.

Good distribution practice (GDP) guidelines emphasize that "regardless of the transport mode, it is important to ascertain that transported product have not been exposed to conditions that may compromise their quality and integrity." This mandates that monitoring of vial or bag of product during transit, before the product is administered for any fluctuations such as the temperature, to ensure that it stayed within defined limits. The limits should be set broad enough to allow the desired transport to occur, but at the same time ensuring that the quality of the product is not compromised. The challenging issue is current lack of understanding of how cell therapy "quality" should be defined as many a times, the mode of action of some cell therapies is not clear, such that there are no defined measurement of cell quality. Therapeutic product potency is often unclear and contingent on expression of nonspecific protein markers. Cell therapy shelf-life cannot be determined without a defined potency assay. Also, it is important to understand that temperature is not the lone factor that has an impact on the cells. Mechanical forces have been shown to affect MSC differentiation potential [26] and MSCs have been reported to be sensitive to vibrations that mimicked road transportation of cells suspended in vials under hypothermic conditions [27]. Great reduction in viability has been reported in cells stored at 4°C for up to 144 h when exposed to vibrations. Though this study is not complete as MSCs were stored in basic culture medium rather than cryopreservative or hypothermic storage medium, it, however, highlights the fact that the effect of transportation on products should be further investigated. Delays in transportation and cost of transportation are other challenges that need attention.

An alternative to transportation is onsite production when products are derived from a patient's own cells. In such case, the hospital needs to have a facility with GMPlicensed clean room that complies with complex regulations for onsite production or commercially available modular GMP environments as mentioned in the previous section such as Xvivo system by Biospherix.

Cost of goods challenges and solutions

Cost of goods (COGs) is aggregate of direct costs such as cost of inventory, raw material, quality control, labor, and indirect costs such as facility and equipment. In RM, COGs are the significant drivers of expensive pricing of the therapeutic drug. Kymriah, a CAR-T therapy chimeric antigen receptor (CAR) T-cell therapy launched by Novartis, has a price tag of US $475,000; Yescarta, another CAR-T therapy by Kite, has a slightly lower price tag of $373,000; and Luxturna, gene therapy by Spark, has a price tag of $425,000 per eye. When you predict the affordability of these therapies, its reach to common man is a very challenging affair. One way to make these therapies affordable is focusing on strategies in reducing the COGs. Harrison et al. [28] presents a COG model that estimates manufacturing capacity required at central facility based on total patient doses guesstimated per year. In this model, COG/doses were estimated using annual cost of material, labor, QC, and indirect costs per annual dose produced. Each of the costs such as material, labor, and QC was further broken down into different categories such as wet consumables (media, buffers, etc.) while labor cost included salary and training. Indirect cost included facility cost, equipment cost, and maintenance cost. It was found that labor cost was one of the largest costs and next is the material cost. As the technology transitions from development to clinical trials to commercial scale, a part of COGs increase due to the increase in requirement for high quality, traceability, and storage, while a part of COGs reduce due to economies of scale. As the number of patients treatable with these therapies increases, the cost per dose will come down. The costs for reagents and consumables

are reduced, as they move into large-scale production to meet growing demand. Though labor is also a big part of the COGs currently, this cost is expected to reduce as the industry automates, simplifies, and combines unit operations. The lack of optimization of manufacturing capacity adds to the overall cost too. Costs will reduce as the effectiveness of these therapies improve, as it would reduce the number of cells needed per dose. Also, use of allogeneic cells for therapy will have a significant impact on cost, as customized and personalized treatments cost more as production will be aimed at one patient. Investing more in automation platform though initially may add up to the capital expenditure, but in the long run, with mega-scale production, it significantly lowers the COGs.

There is a lack of cost data for manufacturing tissue-engineered products, but we would like to highlight one study conducted in the United Kingdom that assessed the cost of tissue-engineered airway transplants [29]. The total costs of treating three patients ranged from $174,420 to $740,500 where the laboratory costs of manufacturing the tissue-engineered product were between 5% and 15% of the total costs, and clinical costs made up the rest. The high clinical costs were due to fact that the patients being treated under the Compassionate Use legislation were severely ill and had significant comorbidities associated with high healthcare costs. The authors estimate that future patients (less comorbidities) could be treated in the range of $105,650–$116,690. Clearly, more studies of this nature on costs associated with manufacturing and clinical care for tissue engineered products are needed.

Business development considerations for regenerative medicine technologies

RM includes cell therapies (non- and genetically modified), tissue-engineered therapeutic products, human cell and tissue products, and combination products having biologic components. RM therapies are transforming how disease, terminal illnesses, and serious disorders are treated and potentially cured with objective to heal and replace organs/cells that have been damaged by age, trauma, or disease. With the signing of the 21st Century Cures Act in December of 2016 and release of two guidance documents "Expedited Programs for RM Therapies for Serious Conditions" and "Evaluation of Devices Used with Regenerative Medicine Advanced Therapies" in February of 2019, FDA has endorsed to advance the field of RM therapeutic innovations, and advocates for development and approval of several regenerative products in the coming years. As compared to the intervention of drugs and chemical in physiological activity inside our body in traditional treatments, cell and gene therapies enable infusion of genes and/or cells for a more effective treatment. Similarly, tissue-engineered product helps to replace injured tissue or organ to restore the physiological function. Despite the great advantages of RM therapies compared to traditional treatments, the high costs of these therapies challenge the reimbursement and insurance coverage. However, it is expected that soon the demand for these therapies will rise as the number of successful clinical trials increase.

Market analysis

As of now, there are 1060 ongoing clinical trials in RM worldwide with 95 cell and gene therapy trials in phase III, suggesting that the number of approved products will soon increase significantly, and with that, demand in the market will rise. FDA has granted 45 RMAT designations since 21st Century Cures Act program inception in December 2016 with 27 being for rare diseases. In January 2019, FDA released statement predicts approval of 20–25 RM therapeutic products per year by 2025 and anticipate receiving more than 200 INDs per year, suggesting a boost in clinical trials. The RM market thus seems promising for a number of strong reasons which include not only the increase in potentially successful clinical trials but also the FDA's strong support to promote developmental work for meeting unmet needs. The RM market is segmented into tissue-engineered products, cell therapies, and gene therapies. In 2019, the tissue-engineered products segment accounted for the largest share in the RM market mainly due to the increasing adoption of tissue-engineered products for the treatment of chronic wounds and musculoskeletal disorders.

Since lot of the products in this market are still in developmental stage, financially, investment into this space is dominated by grants, private investors, and publicly traded stocks. Rising funding for the R&D of RM products and therapies is the major factor to drive the growth of this segment. As these therapies have started to come into market, investors have taken an interest in company's plan on handling large-scale manufacturing. In March of 2019, on the investment outlook panel at ARM's Cell & Gene Investor Day, Aquilo Capital Management Principal Patrick Rivers commented that the issue and challenge of "manufacturing become(s) questions one, two, and three" when making investment decisions.

The demand for RM therapeutic products will rise as the therapies are made available to an increasing number of patients. The field will encounter an increased need for innovative solutions to manufacturing and infrastructure challenges. Stakeholders must develop strategies and come up with innovative solutions to manufacturing hurdles

in large-scale production to attract investor interest and to foster the growth. Combination of efforts of innovative manufacturing strategies, advancement in science of RM products, support of regulatory bodies by providing clear Chemistry, Manufacturing, and Control (CMC) guideline, and cross-sector coordination with strategic business investments will result in significant improvements in the manufacturing environment in coming years. RM therapies have the potential to drastically transform our healthcare systems and improve the lives of hundreds of thousands of patients across the globe in the relatively near future. Stakeholders must now come together to overcome manufacturing challenges of RM therapies to fully leverage the promise of RM. The analysis report by Business Wire indicates that the global RM market was worth $35 billion in 2019 and is estimated grow to over $124 billion by 2025, with a CAGR of 23.3% between this time frame.

Intellectual property protection

Regenerative medicine therapies are widely considered a cornerstone of future health care. These therapies have been recognized as potential treatments for a growing list of indications, particularly those currently lacking any effective treatment options. Employing living cells in treatment of variety of diseases in the areas of oncology, immunology, neurology, rheumatology, and cardiology shows a great promise. The prospects of regenerative medicine therapies to become the most common therapies in near future encourages development of therapeutic products with widespread access for broader benefit. While commercialization of these therapies is greatly in progress, it is crucial that therapies are legally protected to the same degree as traditional treatments. Protecting intellectual property (IP) is another important consideration. Regenerative Medicine intellectual property is mainly protected by patents governed by patent laws and, in many instances, also by data/market exclusivity where clinical data have exclusivity and generic drug makers cannot utilize such clinical data of an innovator for market approval [30]. Another important aspect of intellectual property is regulatory implementation as the related data are protected by data protection/exclusivity and/or a reexamination system to prevent other parties from entering the market [30]. Obtaining and controlling market exclusivity for one of these therapies through patents safeguard an entity's investment, innovation, and IP by discouraging the development and marketing of competing therapies that could threaten market share in the same or overlapping therapeutic spaces [31]. Biologics Price Competition and Innovation Act (BPCIA, 2009) offers developers to take advantage of the 12-year market exclusivity for biologics in addition to patenting their innovations. BPCIA offers developers a regulatory path for products that are biosimilar to, or interchangeable with, previously licensed biologics [31]. However, in the first 4 years, no biosimilar application is accepted by FDA as screened by data presented by the maker of the reference drug. The BPCIA also gives the reference biologic maker 12 years of market exclusivity after approval.

Funding from government in promising research areas enables federal agencies to hold patents to exercise them in enhancing the development and dissemination of technology. To maximize public benefit and widespread access, government could make sure that research tools are obtained in ways to protect basic and future product development. There are few ways that this can be achieved such as defining a strong research exemption to provide third parties access to products and research tools for research purposes without having to obtain permission from the patent holder or enabling negotiation for nonexclusive licenses whenever possible or requiring compulsory licensing under limited and clearly defined circumstances.

Commercialization

To get these regenerative medicine technologies into patients, an effective commercialization strategy needs to be implemented. The manufacturing considerations for any regenerative medicine therapy be it cell therapy, gene therapy, or tissue-engineered constructs, intended to be manufacture at a commercial scale under GMP conditions represent a substantial hurdle as discussed above in manufacturing challenges. Furthermore, developing these therapies requires the use of purpose-designed, accredited good manufacturing facilities, but only few manufacturing facilities can meet those stringent requirements (2019, Routes to Exclusivity in Cell-Based Therapy Development). At the commercialization stage, the companies while focusing on the manufacturing in compliance with regulation should also focus on developing a successful business model and identify strategies based on type of regenerative medicine therapy whether it is autologous or allogeneic. Allogeneic-based therapies are more efficient to commercialize, as multiple batches can be created from one source while autologous therapies are personalized and only one patient can be treated. For expanding the production and to move to commercialization, many businesses partner with Contract Development and Manufacturing Organization (CDMO) and Contract Manufacturing Organization (CMO). Some of the commercial products in market are listed in Table 35.1 that have undergone rigorous regulatory approval process by internationally recognized regulatory agencies such as FDA, EU, Japan PMDA, and SK MFDS.

TABLE 35.1 Highlighted regenerative medicine products.

Product name	Company	Description
Cell-based immunotherapy products		
APCeden	APAC BIOTECH	APCeden is an autologous monocyte-derived mature dendritic cell vaccine. It was approved in India in 2017 for the treatment of prostate cancer, ovarian cancer, colorectal cancer, and non-small-cell lung carcinoma.
Provenge	DENDREON	Autologous cellular immunotherapy for the treatment of asymptomatic or minimally symptomatic metastatic castrate resistant (hormone refractory) prostate cancer. BLA approved by the FDA in 2010.
Immuncell-LC	GC PHARMA	A medicine for anticancer cell immunotherapy, made with T-lymphocyte incubated and activated after extraction from the blood of a patient. Conditionally approved in S. Korea in June 2007.
CreaVax RCC	JW CREAGENE	A dendritic cell-based vaccine intended to treat metastatic renal cell carcinoma for which nephrectomy can be performed. Approved in S. Korea in 2007.
Yescarta	KITE, A GILEAD COMPANY	Yescarta is a CAR T-cell therapy product indicated for the treatment of B-cell malignancies such as non-Hodgkin lymphoma, acute lymphoblastic leukemia, mantle cell lymphoma, chronic lymphoid leukemia, and diffuse large B-cell lymphoma. Approved by US FDA in October 2017. Approved by the EC in August 2018. Approved by Health Canada in February 2019.
Kymriah	NOVARTIS	Kymriah is a CAR T-cell therapy product indicated for the treatment of acute lymphoblastic leukemia, chronic lymphoid leukemia, and diffuse large B-cell lymphoma in patients up to 25 years of age, as approved by US FDA in August 2017, and the treatment of adult patients with relapse/refractory (r/r) large B-cell lymphoma, as approved by US FDA in May 2018. It was also approved for these indications by the EC in August 2018, and by Health Canada in September 2018. It was approved in Japan for the treatment of ALL in February 2019.
Tecartus	KITE, A GILEAD COMPANY	Tecartus is a CAR-T therapy designed to treat mantle cell lymphoma. It was approved in the United States in July 2020.
Gene therapy products		
Imlygic	AMGEN	IMLYGIC is a weakened form of Herpes Simplex Virus Type 1, which is commonly called the cold sore virus, indicated for the local treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with melanoma recurrent after initial surgery. Approved by EMA in December 2015, US FDA in October 2015, Australian TGA in June 2016.
Collategene	ANGES	A gene therapy consisting of an intramuscular injection of plasmid (DNA) coding for hepatocyte growth factor (HGF), intended to grow blood vessels, for the treatment of critical limb ischemia. Approved in Japan in February 2019.
Zolgensma	NOVARTIS GENE THERAPIES	Zolgensma (onasemnogene abeparvovec-xioi) is an adeno-associated virus vector-based gene therapy. Zolgensma was approved by the FDA for the treatment of pediatric patients less than 2 years of age with spinal muscular atrophy (SMA), including those who are presymptomatic at diagnosis, in May 2019. The therapy was approved in Japan in March 2020. It was approved by the EC for certain forms of SMA in May 2020.
Zynteglo	BLUEBIRD BIO	Zynteglo is an ex vivo lentiviral-based gene therapy used to provide a functional copy of the β-globin gene into a patient's hematopoietic (blood) stem cells. Approved by the EMA in May 2019.
Strimvelis	GSK	Strimvelis is an ex vivo stem cell gene therapy which uses retroviral vector encoding adenosine deaminase gene transfer into hematopoietic stem/progenitor cells. Strimvelis is indicated for the treatment of adenosine deaminase severe combined immune deficiency. Approved by the EMA in May 2016.
Gendicine	SHENZHEN SIBIONO GENETECH	A recombinant adenovirus engineered to express wild-type-p53 (rAd-p53), designed to treat patients with tumors which have mutated p53 genes. Gendicine is the first gene therapy product approved for clinical use in humans, approved in China in 2003.
Luxturna	SPARK THERAPEUTICS	LUXTURNA is an adeno-associated viral vector gene therapy indicated for the treatment of RPE65-mediated inherited retinal dystrophies. Approved by US FDA in December 2017; approved by the EMA in November 2018; approved by Health Canada in October 2020.

Continued

TABLE 35.1 Highlighted regenerative medicine products—cont'd

Product name	Company	Description
Cell therapy products		
Queencell	ANTEROGEN	An autologous mesenchymal stem cell for the treatment of connective tissue disorders. Approved in S. Korea in 2010.
Cupistem	ANTEROGEN	An autologous adipose-derived mesenchymal stem cell treatment to reduce inflammation and regenerate damage joint tissues, indicated for the treatment of Crohn's fistula. Approved for marketing by South Korea's Food and Drug Administration in July 2012.
KeraHeal	BIOSOLUTIONS	An autologous keratinocyte-based cell therapy for the treatment of second-degree burns. Approved in S. Korea in 2006.
KeraHeal-Allo	BIOSOLUTIONS	A hydrogel-type allogeneic keratinocyte therapy product for the treatment of second-degree burns. Approved in S. Korea in 2015.
HPC Cord Blood	BLOODWORKS	Approved by the US FDA in January 2016 for use in unrelated donor hematopoietic progenitor cell transplantation procedures in conjunction with an appropriate preparative regimen for hematopoietic and immunologic reconstitution in patients with disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment.
Holoclar	CHIESI FARMACEUTICI	Holoclar is a cell therapy based on autologous cultures of limbal stem cells. It regenerates a functional corneal epithelium allowing recovery of visual acuity. Holoclar is indicated for the treatment of moderate-to-severe limbal stem cell deficiency due to ocular burns. Granted conditional marketing authorization by the European Commission in February 2015.
CLEVECORD/HPC Cord Blood	CLEVELAND CORD BLOOD CENTER	Approved by the US FDA in 2016 for use in unrelated donor hematopoietic progenitor cell transplantation procedures in conjunction with an appropriate preparative regimen for hematopoietic and immunologic reconstitution in patients with disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment.
Neuronata-r	CORESTEM	An autologous bone marrow mesenchymal stem cell therapy approved in S. Korea in 2014 for the treatment of ALS.
Cellgram-AMI	FCB PHARMICELL	An autologous intracoronary bone marrow-derived mesenchymal stem cell injection for the treatment acute myocardial infarction. Approved for marketing by South Korea's Food and Drug Administration in July 2011.
LaViv (Azficel-T)	FIBROCELL TECHNOLOGIES	LaViv is an autologous fibroblast-based cell therapy. Approved by the US FDA in January 2011 for improvement of the appearance of moderate-to-severe nasolabial fold wrinkles in adults.
TEMCELL	JCR PHARMACEUTICALS CO LTD, LICENSEE OF MESOBLAST LTD	TEMCELL is an allogeneic mesenchymal stem cell product indicated for the treatment of acute radiation injury, chronic obstructive pulmonary disease, Crohn's disease, graft-versus-host disease, Type I diabetes and myocardial infarction. Fully approved by the Japanese Ministry of Health, Labour and Welfare in October 2015 and conditionally approved in Canada & New Zealand (also known at Prochymal).
HPC, Cord Blood	MD ANDERSON CORD BLOOD BANK	Approved by the US FDA in June 2018 for use in unrelated donor hematopoietic progenitor cell transplantation procedures.
Cartistem	MEDIPOST	A cellular therapeutic agent containing allogeneic human umbilical cord blood-derived mesenchymal stem cells, indicated for the treatment of knee cartilage defects such as traumatic articular cartilage, degenerative arthritis and rheumatoid arthritis. Approved for marketing by South Korea's Ministry of Food and Drug Safety in January 2012.
Stemirac	NIPRO CORP	A mesenchymal stem cell therapy approved in Japan in December 2018 for the treatment of spinal cord injury.
CureSkin	S. BIOMEDICS	An autologous dermal fibroblast cell-based therapy approved in S. Korea in 2010 for the treatment of depressed acne scars.

TABLE 35.1 Highlighted regenerative medicine products—cont'd

Product name	Company	Description
Hemacord/ ALLOCORD/HPC Cord Blood	SSM CARDINAL GLENNON CHILDREN'S MEDICAL CENTER	The US FDA's only approved stem cell product to date, a cord blood-derived product used for specified indications in patients with disorders affecting the body's blood-forming system. For use in unrelated donor hematopoietic progenitor cell transplantation procedures in conjunction with an appropriate preparative regimen for hematopoietic and immunologic reconstitution in patients with disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment. Approved in 2011, name change to HPC Cord Blood in 2013.
Stempeucel	STEMPEUTICS RESEARCH PVT	An ex vivo-cultured adult allogeneic mesenchymal stromal cell therapy for the treatment of critical limb ischemia. Conditionally approved in India in 2017.
Kaloderm	TEGO SCIENCE	An allogeneic cell therapy for deep second-degree burns and diabetic foot ulcers. Approved in S. Korea for burns in 2005 and diabetic foot ulcers in 2010.
Rosmir	TEGO SCIENCE	Autologous cell therapy approved in S. Korea in 2018 for the treatment of under eye wrinkles.
Alofisel	TIGENIX	An allogeneic stem cell therapy to treat complex perianal fistulas in patients with Crohn's disease. Received EU marketing authorization in March 2018.
Tissue-engineered products		
Epicel	VERICEL	A permanent skin replacement product grown from a patient's own skin cells. The autologous keratinocytes are co-cultured with irradiated murine cells to form cultured epidermal autografts (CEA). Epicel is indicated for treatment of deep dermal or full thickness burns. Epicel has been used in the USA and other countries since 1988; approved in the United States in 2007 as a Humanitarian Use Device (HUD) under a Humanitarian Device Exemption (HDE).
Ossron		An autologous bone cell implantation for the treatment of bone defects in patients caused by degeneration, drugs, intense physical stress, diet, genetics, obesity, smoking, alcohol or disease. Approved in S. Korea in 2009, approved in India in 2017.
MACI	AUTOLOGOUS CULTURED CHONDROCYTES ON A PORCINE COLLAGEN MEMBRANE/VERICEL	An autologous cellularized scaffold product, indicated for the repair of single or multiple symptomatic, full-thickness cartilage defects of the knee with or without bone involvement in adults. Approved by the US FDA in December 2016.
Omnigraft	INTEGRA	An advanced bilayer dermal regeneration matrix for the treatment of diabetic foot ulcers. Approved by the FDA in 2016.
CardioCel	ADMEDUS	A cardiovascular scaffold which facilitates endogenous stem cells and other cells to regenerate and repair damaged tissue for the treatment of cardiovascular abnormalities. Marketed in the United States under 510(k) clearance as of 2014; marketed in Europe under CE Mark as of 2013; received medical device license in Canada in 2014; approved in Singapore in 2015.
Spherox (formerly Chrondosphere)	CO.DON AG	A product containing spheroids of human autologous chondrocytes for use in cartilage defects. Marketed in Europe as of 2017; marketed in Germany since 2007.
ReNovaCell	AVITA MEDICAL	An autologous cell harvesting device that enables to create a regenerative epithelial suspension using the sample of the patient's skin for the treatment of skin discoloration. Marketed in Europe as of 2016.
Vergenix FG	COLLPLANT	A device composed of homogenous human recombinant type I collage for use in wound treatment. Marketed in Europe as of 2016.
Vergenix-STR	COLLPLANT	A matrix made of collagen type I for use in the treatment of connective tissue disorders. Marketed in Europe as of 2016.
ReGenerCel	AVITA MEDICAL	An autologous cell harvesting device that enables to create a regenerative epithelial suspension using the sample of the patient's skin for the treatment of ulcers. Marketed in Europe as of 2015.
Aurix	NUO THERAPEUTICS	A biodynamic hematogel composed of platelet-rich plasma gel prepared from a small sample of a patient's own platelets and plasma as a catalyst for healing indicated for the treatment of wounds. Marketed in the United States under Section 361 for the Platelet Rich Plasma and under 510(k) clearance as of September 2007.

Continued

TABLE 35.1 Highlighted regenerative medicine products—cont'd

Product name	Company	Description
Hyalograft 3D	CHA BIO&DIOSTECH CO LTD	A cell therapy which cultivates autologous skin fibroblasts in 3D scaffolds formed of hyaluronic acid derivatives for the treatment of diabetic foot ulcers. Conditionally approved in S. Korea in July 2007.
Holloderm	TEGO SCIENCE	A cultured epidermal autograft composed by culturing autologous keratinocytes. It is transplanted to the wound and aids in regeneration of the dermis and develops into new skin. Holloderm is indicated for the treatment of skin disorders such as burns, vitiligo, nevi, and scars. Approved in S. Korea in 2002.
Heart Sheet	TERUMO BCT	An autologous skeletal myoblast preparation approved in Japan in 2015 for the treatment of patients with serious heart failure.
Transcyte	ORGANOGENESIS	A tissue-engineered skin substitute made from a nylon mesh and a silastic semipermissible and biocompatible layer for the treatment of Epidermolysis Bullosa. Approved by the FDA in 1998, acquired by Organogenesis from Shire in 2014.
Apligraf	ORGANOGENESIS, INC. & NOVARTIS AG	A bi-layered living skin substitute made from a dermal layer of human cells (fibroblasts) in a bovine type I collagen and an overlying cornified epidermal layer of living human keratinocytes. Apligraf is indicated for the treatment of chronic venous leg ulcers and diabetic foot ulcer. Approved by US FDA in July 1998 and June 2000, respectively.
Dermagraft	ORGANOGENESIS	A dermal substitute used to help in the wound closure of diabetic foot ulcers. It is made from human cells (fibroblasts), placed on a dissolvable mesh material. Organogenesis acquired Dermagraft from Shire in 2014. Approved by US FDA in September 2001.
JACC	J-TEC	A combination product of autologous cultured chondrocytes and collagen gel approved in Japan in 2012 for the alleviation of clinical symptoms of traumatic cartilage defect or osteochondritis dissecans of the knee.
JACE	J-TEC	An epidermal cell sheet produced from keratinocytes isolated from a patient's own skin tissue, approved in Japan in 2007 for use in patients with deep dermal and full-thickness burns covering 30% or more of the total body surface area; approved in Japan in 2016 to treat patients following the excision of giant congenital melanocytic nevi to facilitate the closure of the wound.
Ortho-ACI	ORTHOCELL	Ortho-ACI is an autologous chondrocyte-based cellularized scaffold to treat symptomatic defects of the articulating cartilage of the joints, predominately the knee and ankle. Approved in Australia 2017.
Novocart 3D	AESCULAP BIOLOGICS	The product consists of three-dimensional collagen-chondroitin sulfate scaffolds with embedded autologous chondrocytes in a cell suspension. Marketed in the EU for articular cartilage repair since 2003.

Alliance for Regenerative Medicine (https://alliancerm.org/available-products/).

Storage logistics

Bringing technologies that can assist with storage logistics across different temperature ranges is extremely important. While challenges of production at large scale and commercialization are addressed, it becomes equally important to focus on the storage of the final products. Most regenerative medicine therapeutic products must be stored below −150°C, a temperature band that brings unique and critical requirements. An alliance of regenerative medicine industry with cold storage industry can aid in providing the exact solution based on the product need. Understanding the product behavior at different temperatures in long term and having stability data become of extreme essence in bulk production.

Cost of final commercial product and value proposition

We identified one case study on cost of manufacturing and transplanting a tissue-engineered product. This final cost which will include both aspects needs to be considered along with the benefits that these regenerative medicine products will bring to our patients and society. Especially with the current COVID-19 pandemic, there will be a tremendous reduction in quality of life for many post COVID-19 if regenerative medicine therapies are not available to restore function of damaged organs.

Reimbursement

With the high cost of these therapies, reimbursement strategies are to be explored to give access to all those in need. The cost of a regenerative medicine therapy has a broad range with a simple stem cell treatment costing anywhere from $5000 to $25,000, and varies globally based on region where treatment is done. Getting insurance to provide reimbursement for regenerative medicine products is possibly a greater challenge than getting regulatory approval. Reimbursement aspect can be a decisive factor in the success or failure of small businesses or product reaching the market. However, success stories of treatment with growing demand would call for attention by healthcare insurance industry to consider inclusion of regenerative medicine treatments in their reimbursement plans.

Conclusions and future vision development considerations

There is no doubt that applying robust manufacturing strategies to regenerative medicine therapies and technologies will be one of the key advances to enable this next generation of healthcare to become a reality. The more that raw materials and processes can be standardized, qualified, controlled, and optimized, the more robust the RM products such as cells and tissue-engineered construct will be which will translate into improved clinical benefit for patients. Also, identifying the risk areas, devising and implementing solutions early on in the developmental stage to develop robust-manufacturing strategies for these therapies and technologies will reduce cost and accelerate process development. As these treatments and technologies become more widespread, adoption of them will be easier. There is a bright future for regenerative medicine and a robust manufacturing process will definitely assist in making this future a reality.

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Regenerative Nephrology

Second Edition

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Since the publication of the first edition of this book in 2010, an explosion of spectacular discoveries in the field of regeneration has compelled the current revisit of the field of Regenerative Nephrology. This second edition features subjects as diverse as age and gender influencing regenerative processes; mechanisms and pathways of premature cell senescence affecting kidney regeneration; the ways intrinsic regenerative processes can become subverted by noxious stressors eventuating in disease progression; novel mechanistic and engineering efforts to recreate functional kidney or its component parts; cell reprogramming and reconditioning as emerging tools of future regenerative efforts; and effects of various biologicals on kidney regeneration. These newer additions to the armamentarium of Regenerative Medicine and Nephrology have become an integral part of the second edition of the book. Cutting-edge investigations are summarized by the constellation of the most experienced contributing authors coming together from around the world under the umbrella of the second edition.

Key new features

- A significant expansion of section on induced pluripotent cells and trajectories of their differentiation. This is followed by mechanisms and modalities of cell reprogramming for therapeutic purposes.
- A new section on tissue engineering of the kidney to be of interest to nephrologists and urologists.
- An entire section dedicated to causes of regenerative failure with the emphasis on recent discoveries of senescent cells in kidney disease, pathologic effects of senescent cells, advents in senotherapies and rejuvenation therapies.
- A vastly expanded section on pharmacotherapies promoting kidney regeneration, trials of engineered organs, manufacturing in regenerative medicine and smooth transition to the clinical trials, with an update on some ethical issues.

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